"ISOLATION AND BIOLOGICAL SCREENING OF ENDOPHYTIC FUNGI FROM *NERIUM OLEANDER* LINN. (APOCYNACEAE)"

A Dissertation submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY, CHENNAI.

In partial fulfillment of the requirement for the

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MASTER OF PHARMACY
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Submitted by

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SEPTEMBER – 2008

DEDICATED TO MY MOTHER

CERTIFICATE

This is to certify that the work embodied in this thesis entitled, **"ISOLATION AND BIOLOGICAL SCREENING OF ENDOPHYTIC FUNGI FROM *NERIUM OLEANDER* LINN (APOCYNACEAE)"** submitted to The TamilNadu Dr. M.G.R. Medical University, Chennai, was carried out by **Mr. KATHIRIYA MAYUR VALLABHBHAI,** Department of Pharmaceutical Biotechnology, Nandha College of Pharmacy, Erode-52 for the partial fulfillment for the award of degree of Master of Pharmacy in Pharmaceutical Biotechnology under my supervision.

This work is original and has not been submitted in part or full for any other degree or diploma of this or any other university.

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DECLARATION

The work presented in this thesis entitled **"ISOLATION AND BIOLOGICAL SCREENING OF ENDOPHYTIC FUNGI FROM *NERIUM OLEANDER* LINN (APOCYNACEAE)"** was carried out by me in the Department of Pharmaceutical Biotechnology, Nandha College of Pharmacy, Erode-52 under the direct supervision of **Mr. K. Kamalakannan, M.Pharm.,** Asst. Professor, Dept. of Pharmaceutical Biotechnology, Nandha College of Pharmacy, Erode-52

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KATHIRIYA MAYUR VALLABHBHAI

CONTENTS

ABBREVATIONS

%	Percentage
±	Positive or negative
β	Beta
κ	Kappa
µg/ml	Microgram per milliliter
cfu	Colony forming units
CHF	Congestive heart failure
DM	Diabetes mellitus
EDTA	Ethylene diamine tetra acetic acid
g	Gram
h	Hour
mg/ml	Milligram per milliliter
MIC	Minimal inhibitory concentration
min	Minute
mL	Milliliter
mm	Millimeter
nm	Nanometer
nmol	Nanomol
^{o}C	Degree Celsius
rpm	Revolutions per minute
SDA	Sabouraud dextrose agar
SRB	Sulphorodamine B
temp	Temperature
UV	Ultraviolet
w/v	Weight by volume

1. INTRODUCTION

1.1 **ANTIFUNGAL DRUGS**[1-3]

The use of chemical or synthetic agents with antimicrobial activity (as inhibitors, growth reducers or even inactivators) is one of the oldest techniques for controlling microorganism growth. In the United States, only 10 antifungal drugs are currently approved by the Food and Drug Administration (FDA) for the therapy of systemic fungal infections. As shown in table, these drugs belong to 3 principle classes: polyenes, pyrimidines, and azoles. Drugs that belong to other classes are also approved as topical antifungal drugs.[1, 2]

Although conventional amphotericin B (Fungizone) remains the standard therapy for many invasive or life-threatening mycoses, this polyene drug is associated with significant toxicity, including infusion-related events, such as chills, fever, headache, nausea and vomiting, and dose-limiting nephrotoxicity. In addition, the clinical efficacy of amphotericin B in some settings (e.g., mold disease such as invasive aspergillosis in severely immunocompromised patients) is suboptimal. Consequently, 3 new lipid formulations of amphotericin B (amphotericin B lipid complex, amphotericin B cholesteryl sulfate, and liposomal amphotericin B) have been developed and recently approved by the FDA. These lipid formulations offer several advantages over conventional amphotericin B, including increased daily dose of the parent drug (up to 10-fold), high tissue concentrations in the primary reticuloendothelial organs (lungs, liver, and spleen), decrease in infusion-associated side effects (especially liposomal amphotericin B), and marked decrease in nephrotoxicity.

Although the therapeutic : toxic ratio of these compounds is clearly improved, superiority in clinical efficacy has not been definitively established in head-to-head comparative trials, either a lipid formulation versus conventional amphotericin B or 1 lipid formulation versus another lipid formulation.

Moreover, these lipid formulations of amphotericin B are considerably more expensive than conventional amphotericin B, ranging from 10- to 20-fold higher in cost per dose. In addition, the optimum daily or total dose of these lipid compounds has not been established. Accordingly, unanswered questions and controversy abound about several issues relating to these 3 lipid agents.

- **Diffusion assays**[3]

Diffusion assays are carried out on a solid medium, usually an agar medium, which is suitable for growth of the test microorganisms. The compound to be assayed is allowed to diffuse through the medium in a radial fashion from a pad/cap so that the adjacent growth of the test microorganism is either depressed, as with an antibiotic or stimulated, as with a growth factor. The diameter of these areas reflects the concentration of the compound being assayed, and it is compared with similar zones produced by various known concentration of standard or reference compound. The zone diameter of the standard are plotted against logs of the concentration used and the linear portion of this standard curve is used for determining the actual concentration of the sample being assayed.[3]

There are two types of diffusion assay and although somewhat similar, each has its particular advantages. In the cylinder method, the portion of the antibiotic solution or other fermentation product diffuses from a reservoir or cylinder into the surrounding agar while in the paper disc method only a defined amount of fermentation solution, such as 0.1ml, is applied to the disc. A standardized amount of agar medium, perhaps 10ml is placed in petri pleats and allowed to harden. As soon as this base layer is hard a standardized amount (usually 5ml) of the same or a different agar medium inoculated with a test microorganism is added above the base layer and allowed to harden to form the “seeded

agar" layer. Several small metals, glazed porcelain or glass cylinders are set on the agar surface. A slight preheating of these cylinders helps to seal them to the agar. The number of cylinders used per plate depends on the expected sizes of the zones, since the zones should not overlap. In fact, it is often necessary to dilute the samples to hold down the sizes of the zones. The cylinders are filled with appropriate dilutions of the solutions to be assayed or with solution containing known concentration of the reference compound and the plates are incubated for a specified period of time at constant temperature. The diameters of the zones of stimulated/reduced growth are then measured in mm and the concentration in the solutions under assay are determined by comparison with a standard, curve prepared from the inhibition/stimulation zone data for the standards.

To obtain valid and reproducible results, each sample of unknown compound and each concentration of reference compound should be replicated several times on different plates so that average values can be calculated. Each assay plate also should contain a cylinder with at least one concentration of standard solution in addition to the fermentation samples, because variability in zone sizes is likely to be greater when comparing values from different plates than when comparing values from the same plate.[3]

1.2 HEART FAILURE[4-10]

Congestive heart failure (CHF) is a major contributor to morbidity and mortality worldwide. There are approximately 5 million established cases of heart failure in the United States alone; a similar number of patients have asymptomatic left ventricular dysfunction and are therefore at risk to develop CHF. Heart failure accounts for more than half a million deaths annually in the United States; mortality in patients with advanced heart failure exceeds 50% at 1 year.

Heart failure develops when the heart can no longer provide adequate blood flow and/or pressure to meet the body's demands. This failure triggers countermeasures, including the retention of salt and water by the kidneys, the stimulation of the body's organs by neurohormones, and the

activation of intracellular signaling cascades in the heart and vasculature that alter cellular and organ morphology and function. Such 'compensatory' responses can initially offset reduced cardiac performance, but they become key 'co-conspirators' in the disease process, ultimately increasing the likelihood of organ failure and worsening clinical prognosis. Although individuals with heart failure have some symptoms in common, including fatigue, shortness of breath and fluid retention, the clinical presentation of heart dysfunction is heterogeneous. About half of all individuals have contractile failure and a dilated heart (that is, systolic heart failure), and the other half seem to have normal contraction and a non-dilated, but often hypertrophied, heart (that is, heart failure with a preserved ejection fraction (HFpEF), which has also been termed diastolic heart failure). HFpEF is increasing in prevalence worldwide but remains understudied.[4]

- **Diagnosis**

Diagnosis is the first essential step in the provision of good management of heart failure. Diagnosis of heart failure by clinical means alone may be fairly accurate in advanced heart failure but will only be correct in about 50% of milder cases; little better than guessing. In at risk groups, such as those who have had a myocardial infarction, symptoms alert the clinician to a possible diagnosis of heart failure which must be confirmed by further investigation. The echocardiogram is the single most useful diagnostic tool for heart failure in widespread use at the present.[5]

- **Therapy**

In recent decades, therapy for heart failure has undergone several large shifts, moving from a focus on haemodynamics to a focus on the targeting of specific disease mechanisms.

One such shift is that neurohormonal stimulation is no longer viewed as always having beneficial effects but as often worsening heart failure and it is therefore a process that should be blocked. For example, arterial vasodilators have long been known to have therapeutic effects through

reducing the workload of the heart; however, more recently, inhibitors of the renin–angiotensin–aldosterone system were found to be more effective than arterial vasodilators, even though they have similar effects on workload. An even more remarkable example is the use of β-blockers, which antagonize the activation of β-adrenergic receptors (β-ARs) on cardiomyocyte by the sympathetic nervous system. Although β-blockers were long considered to be contra indicated for heart failure, they were ultimately shown to improve outcome and are now established therapeutics.

A second shift has been to avoid treatments designed to stimulate muscle in the weakened heart. Although such therapy helped in the short term, it was generally found to be detrimental when carried out long term.

A third shift is that a 'bionic' era is now dawning, in which implantable devices controlled by microprocessors can deliver therapy, monitor disease and rescue a patient from sudden cardiac death. Since the turn of the twenty-first century, these devices have had the most impact of any new treatment for heart failure.[6]

The ability to replace damaged myocardium by the use of stem cell therapy offers a new and potentially exciting treatment modality, but its benefits are at present far from established. The current state of cardiac stem cell therapy and its future has been reviewed briefly.[7]

The pharmacological treatment of heart failure for many years was limited to the use of digitalis glycosides and diuretics. Although *digitalis* has been supplanted by therapies that provide a mortality benefit (*e.g.,* ACE inhibitors), the clinical and investigative uses of the cardiac glycosides have informed approaches to therapy and drug design and development. Similarly, while diuretics do not offer a mortality benefit, volume overload clearly remains a central component of the clinical syndrome and is often the factor that leads to initial diagnosis or to hospitalization for treatment of acute exacerbations.

The current approach to therapy for CHF involves preload reduction, afterload reduction, and enhancement of ionotropic state. A variety of vasodilators will reduce preload and afterload. Although

a vasodilator's more prominent effect may be the reduction of either preload or afterload, most agents affect both, to differing extents.[4]

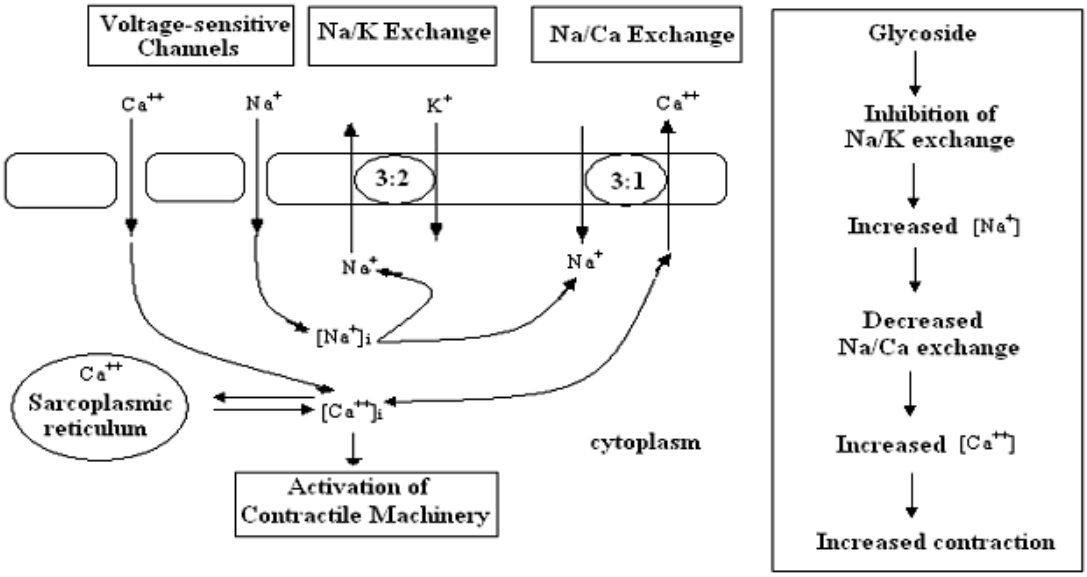
- **Cardiac Glycosides**

The beneficial effects of cardiac glycosides in the treatment of heart failure have been attributed to a positive ionotropic effect on failing myocardium and efficacy in controlling the ventricular rate response to atrial fibrillation. The cardiac glycosides also modulate autonomic nervous system activity, and it is likely that this mechanism contributes substantially to their efficacy in the management of heart failure. The cardiac glycosides possess a common molecular structure, a steroid nucleus containing an unsaturated lactone at the C17 position, and one or more glycosidic residues at C3. However, the advent of alternative therapies that both palliate symptoms and improve survival has led to a more limited role for the cardiac glycosides in the pharmacotherapy of congestive heart failure. Only digoxin (LANOXIN, LANOXICAPS) is in widespread clinical use today[4].

- **Mechanisms of Action: *Inhibition of Na^+, K^+-ATPase***

All cardiac glycosides are potent and highly selective inhibitors of the active transport of Na^+ and K^+ across cell membranes. This biological effect is accomplished by binding to a specific site on the β subunit of Na^+, K^+-ATPase, the cellular Na^+ pump. The Na^+, K^+-ATPase is a heterodimeric (designated, α- and β- subunits) transmembrane protein complex that serves as the receptor for cardiotonic steroids such as digitalis. Na^+, K^+-ATPase and other P2-type ATPases use the hydrolysis of ATP to drive ion transport across cell membranes. This enzyme also plays an important role in the regulation of blood pressure, and it specifically mediates adrenocorticotropic hormone (ACTH)-induced hypertension in mice. The binding of cardiac glycosides to Na^+, K^+-ATPase and inhibition of the cellular ion pump is reversible and entropically driven.[8-10]

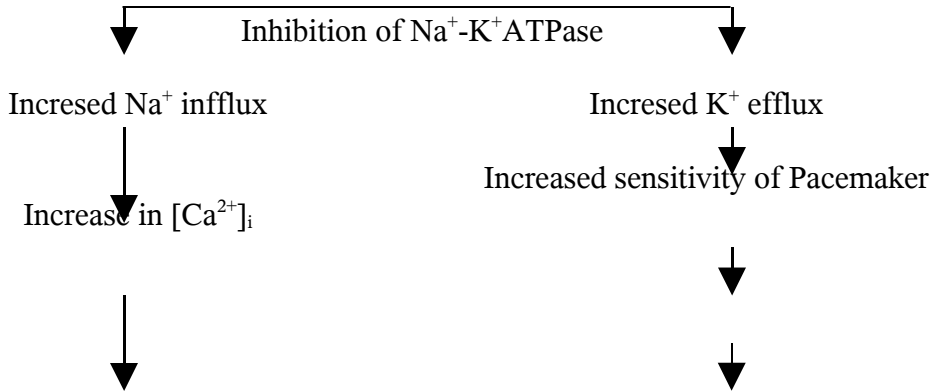
Figure 1.1: Mechanism of action of Cardiac Glycoside[10]



- **Electrophysiological Actions**

At therapeutic serum or plasma concentrations (*i.e.,* 1 to 2 ng/ml), cardiac glycoside decreases automaticity and increases maximal diastolic resting membrane potential in atrial and AV nodal tissues, due to an increase in vagal tone and a decrease in sympathetic nervous system activity. In addition, there is prolongation of the effective refractory period and decreased conduction velocity in AV nodal tissue. These aggregate effects may cause sinus bradycardia or arrest and/or prolongation of AV conduction or higher-grade AV block. At higher concentrations, cardiac glycosides can increase sympathetic nervous system activity and directly affect automaticity in cardiac tissue, actions that contribute to the genesis of atrial and ventricular arrhythmias. Increased intracellular Ca^{2+} loading and increased sympathetic tone increase the spontaneous (phase 4) rate of diastolic depolarization as well as delayed afterdepolarizations that may reach the threshold for generation of a propagated action potential.[4, 10]

Figure 1.2: Action of Cardiac Glycoside on Heart[10]



Slows AV conduction

Increase in force of contraction (Helps in CHF)

Prolongation of depolarization (Helps in arrythmias)

This simultaneous nonuniform increase in automaticity and depression of conduction in HIS-Purkinje and ventricular muscle fibers produces an electrophysiologic substrate that predisposes to serious ventricular arrhythmias, including ventricular tachycardia and ventricular fibrillation.[10]

- **Clinical Use of Cardiac glycoside in Heart Failure**

The cardiac glycosides have long been used in the treatment of CHF. For a century, however, there has been controversy surrounding the efficacy of cardiac glycosides in the treatment of patients with heart failure who are in sinus rhythm.

The PROVED (Prospective Randomized study Of Ventricular failure and Efficacy of Digoxin) and RADIANCE (Randomized Assessment of Digoxin on Inhibition of Angiotensin Converting Enzyme) trials examined the effects of withdrawal of cardiac glycoside in stable patients with mild-to-moderate heart failure (*i.e.* NYHA Class II and III) and systolic ventricular dysfunction (left ventricular ejection fraction <0.35%). All patients studied were in normal sinus rhythm. Withdrawal of cardiac glycoside resulted in a significant worsening of heart failure symptoms in patients who received placebo compared with patients who continued to receive active drug.[4]

- **Use of Cardiac glycoside in Clinical Practice and Monitoring of Serum Levels**

It is now recommended that cardiac glycoside be reserved for patients with heart failure who are in atrial fibrillation, or for patients in sinus rhythm who remain symptomatic despite maximal therapy with ACE inhibitors and β adrenergic receptor antagonists. The latter agents are viewed as first-line therapies on the basis of the proven mortality benefit. Most studies suggest that the maximal

increase in contractility is apparent at serum levels of cardiac glycoside around 1.4ng/ml or 1.8 nmol. The neurohormonal benefits of cardiac glycoside may occur at lower serum levels, between 0.5 and 1 ng/ml; higher serum concentrations are not associated with further decreases in neurohormonal activation or with increased clinical benefit. A retrospective subgroup analysis of the DIG trial suggested that the risk of death was greater with increasing serum concentrations, even at values that were within the traditional therapeutic range. Many authorities therefore advocate maintaining cardiac glycoside levels below 1 ng/ml.

In summary, cardiac glycoside is no longer viewed as a first-line agent in the treatment of congestive heart failure. Despite this fact, it should be emphasized that cardiac glycoside, unlike virtually all other ionotropic agents studied to date, does not have an adverse impact on mortality in CHF. The ionotropic effects of digitalis were first identified more than 200 years ago and, despite the development of newer pharmacological agents such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor antagonists and b-blockers, the cardiac glycosides remains first-line agents in the treatment of CHF and supraventricular arrhythmias. Thus, cardiac glycoside is a therapeutic option in patients who remain symptomatic despite treatment with agents that improve survival. Cardiac glycoside may be unique among ionotropic drugs by virtue of its neurohumoral effects, which include attenuation of sympathetic activation and reduction of renin release.[4]

- **Cardiac glycoside Toxicity**

Vigilance for and early recognition of disturbances of impulse formation, conduction, or both are critically important. Among the more common electrophysiological manifestations are ectopic beats of AV junctional or ventricular origin, first-degree AV block, an excessively slow ventricular rate response to atrial fibrillation, or an accelerated AV junctional pacemaker. These often require only a dosage adjustment and appropriate monitoring. Sinus bradycardia, sinoatrial arrest or exit block, and second- or third-degree AV conduction delay usually respond to atropine, although temporary

ventricular pacing may be necessary. Potassium administration should be considered for patients with evidence of increased AV junctional or ventricular automaticity, even when the serum K^+ is in the normal range, unless high-grade AV block also is present. Lidocaine or phenytoin, which has minimal effects on AV conduction, may be used for the treatment of worsening ventricular arrhythmias that threaten hemodynamic compromise. Electrical cardioversion carries an increased risk of inducing severe rhythm disturbances in patients with overt digitalis toxicity and should be used with particular caution. Note, too, that inhibition of the Na^+, K^+-ATPase activity of skeletal muscle can cause hyperkalemia.[4]

- **Anticardiac glycoside Immunotherapy**

An effective antidote for life-threatening cardiac glycoside or *digitoxin* toxicity is available in the form of anticardiac glycoside immunotherapy with purified Fab fragments from ovine anticardiac glycoside antisera (DIGIBIND). A full neutralizing dose of Fab based on either the estimated total dose of drug ingested or the total body cardiac glycoside burden can be administered intravenously in saline solution over 30 to 60 minutes.[4]

1.3 DIABETES[11-14]

Diabetes mellitus (DM) is a group of syndrome characterized by hyperglycemia; altered metabolism of lipids, carbohydrates and proteins; and an increased risk of complications from vascular disease. Most patients can be classified clinically as having either type 1 DM (formerly known as insulin-dependent diabetes mellitus or IDDM) or type 2 DM (formerly known as non-insulin-dependent diabetes mellitus or NIDDM).

The incidence of each type of diabetes varies widely throughout the world. In the United States, about 5% to 10% of all diabetic patients have type 1 DM, with an incidence of 18 per 100,000 inhabitants per year, the remaining 90% having type 2 DM. The incident rates of type 2 DM increase with age, with a mean rate of about 440 per 100,000 per year by the sixth decade in males in the United States.

There are more than 125 million persons with diabetes in the world today, and by 2010 this number is expected to approach 220 million. Both type 1 and type 2 DM are increasing in frequency. The reason for the increase of type 1 DM is not known. The genetic basis for type 2 DM cannot change in such a short time; thus other contributing factors, including increasing age, obesity, sedentary lifestyle, and low birth weight, must account for this dramatic increase. In addition, type 2 DM is being diagnosed with remarkable frequency in preadolescents and adolescents. Up to 45% of newly diagnosed children and adolescents have type 2 DM. Direct costs for medical services and indirect costs because of lost productivity due to diabetes approached 132 billion dollars in 2002[11].

Undiagnosed diabetes remains an important health problem with approximately one in five male diabetics undiagnosed in 1999–2002.

Table 1.2: Different Forms of Diabetes Mellitus[11]

General—genetic and other factors not precisely defined

Type 1 diabetes mellitus (formerly called insulin-dependent DM)

- Auto-immune type 1 DM (type 1A)
- Non-autoimmune/idiopathic type 1 DM (type 1B)

Type 2 diabetes mellitus (formerly called non-insulin-dependent DM)

- **Specific—defined gene mutations**
  - Maturity-onset diabetes of youth (MODY)
    - MODY 1 hepatic nuclear factor 4α (*HNF4A*) gene mutations
    - MODY 2 glucokinase (*GCK*) gene mutations
    - MODY 3 hepatic nuclear factor 1α (*TCF1*) gene mutations
    - MODY 4 insulin promoter factor 1 (*IPF1*) gene mutations
    - MODY 5 hepatic nuclear factor 1β (*HNF1* β) gene mutations
    - MODY 6 neurogenic differentiation 1 (*NEUROD1*) gene mutation
    - MODY X unidentified gene mutation(s)
  - Maternally inherited diabetes and deafness (MIDD)
  - Mitochondrial leucine tRNA gene mutations
  - Insulin gene mutations
  - Insulin receptor gene mutations
- **Diabetes secondary to pancreatic disease**
  - Chronic pancreatitis
  - Surgery
  - Tropical diabetes (chronic pancreatitis associated with nutritional and/or toxic factors)
- **Diabetes secondary to other endocrinopathies**
  - Cushing's disease
  - Glucocorticoid administration
  - Acromegaly
- **Diabetes secondary to immune suppression**
- **Diabetes associated with genetic syndromes; *e.g.*, Prader-Willi syndrome**
- **Diabetes associated with drug therapy**

This is far less of a problem than 25 years ago, when almost half of male diabetics were undiagnosed. Although race and ethnic differentials in undiagnosed diabetes were eliminated over the last 25 years, the disparities became larger across other measures of disadvantage such as education. Undiagnosed diabetes is a particularly severe problem among the obese, a group at much higher risk of diabetes onset.[12]

- **Cause**[11, 13, 14]

In certain tropical countries, the most common cause of diabetes is chronic pancreatitis associated with nutritional or toxic factors (a form of secondary diabetes). Very rarely, diabetes results from point mutations in the insulin gene. Amino acid substitutions from such mutations may result in insulin with lower potency or may alter the processing of proinsulin to insulin.[11]

There are genetic and environmental components that affect the risk of developing either type 1 or type 2 DM. There is considerable evidence that type 1 DM involves an autoimmune attack on the pancreatic β cell. Both Fas-dependent and -independent mechanisms are involved in β cell destruction, but interference with the Fas pathway early in disease development may retard or prevent diabetes progression. Studies have determined that the presence of antibodies directed against insulin (IAAs) confers only a small risk for the development of type 1 DM. On the other hand, the presence of high-titer islet-cell antibodies (ICAs) and GAD antibodies or ICAs combined with IAAs confers a very high risk for the development of type 1 DM in first-degree relatives. Individuals with type 1 DM also tend to have antibodies directed toward other endocrine tissues, including the adrenal, parathyroid, and thyroid glands, and have an increased incidence of other autoimmune diseases.[11, 13]

The trigger for the immune response remains unknown. The identification of triggering agents is difficult because autoimmune destruction of pancreatic β cells may occur over a period of many months or several years before the onset of overt disease. In about 10% of new cases of type 1 DM, there is no evidence of autoimmune insulitis. World Health Organization (WHO) therefore subdivides this disease into autoimmune (1A) and idiopathic (1B) subtypes. Whatever be the causes, the final

result in type 1 DM is an extensive and selective loss of pancreatic β cells and a state of absolute insulin deficiency.[11]

Patients with type 2 DM either have a decreased production of insulin by the β cells of the pancreas or a decreased sensitivity of the cells to insulin, making the cells insulin resistant. Although type 2 DM may occur at any age, the disorder occurs most often after the age of 40 years. The onset of type 2 DM is usually insidious, symptoms are less severe than in type 1 DM, and because it tends to be more stable, it is easier to control than type 1 DM. Risk factors included in the screening guidelines have a strong association with diabetes. Hypertension or positive family histories of diabetes can double the risk of having diabetes.

Virtually all forms of DM are caused by a decrease in the circulating concentration of insulin (insulin deficiency) and a decrease in the response of peripheral tissues to insulin (insulin resistance). These abnormalities lead to alterations in the metabolism of carbohydrates, lipids, ketones, and amino acids; the central feature of the syndrome is hyperglycemia.[11, 14]

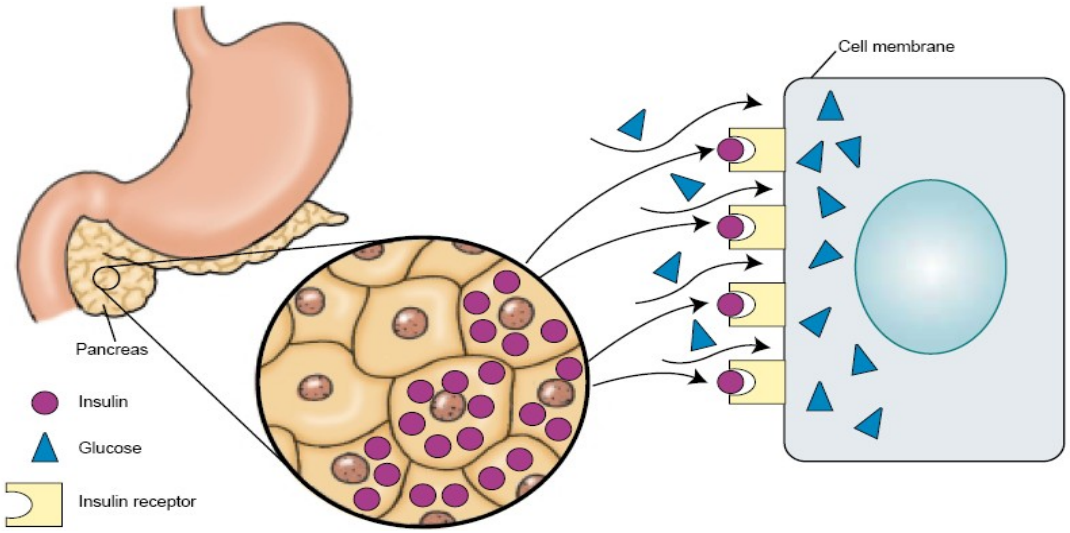
Risk factors for type 2 diabetes include:

- Obesity
- Older age
- Family history of diabetes
- History of gestational diabetes (diabetes that develops during pregnancy but disappears when pregnancy is over)
- Impaired glucose tolerance
- Minimal or no physical activity
- Race/ethnicity (African Americans, Hispanic/Latino Americans, American Indians, and some Asian Americans)[11]

- **Insulin**[11]

Insulin is a hormone manufactured by the β cells of the pancreas. It is the principal hormone required for the proper use of glucose (carbohydrate) by the body. Insulin also controls the storage and utilization of amino acids and fatty acids. Insulin lowers blood glucose levels by inhibiting glucose production by the liver.

Figure 1.3: Normal Glucose Metabolism[11]



Insulin is available as purified extracts from beef and pork pancreas and is biologically similar to human insulin. However, these animal source insulins are used less frequently today than in years past. They are being replaced by synthetic insulins, including human insulin or insulin analogs. Human insulin is derived from a biosynthetic process using strains of *Escherichia coli* (recombinant DNA technique). Human insulin appears to cause fewer allergic reactions than does insulin obtained from animal sources. Insulin analogs, insulin lispro, and insulin aspart are newer forms of human insulin made by using recombinant DNA technology and are structurally similar to human insulin.

- **Indications and Goals for Therapy**[11]

Subcutaneous administration of insulin is the primary treatment for all patients with type 1 DM, for patients with type 2 DM that is not controlled adequately by diet and/or oral hypoglycemic agents, and for patients with postpancreatectomy diabetes or gestational diabetes. In addition, insulin is critical

for the management of diabetic ketoacidosis, and it has an important role in the treatment of hyperglycemic, nonketotic coma and in the perioperative management of both type 1 and type 2 DM. In all cases, the goal is to normalize not only blood glucose but also all aspects of metabolism; the latter is difficult to achieve. Optimal treatment requires a coordinated approach to diet, exercise, and the administration of insulin.

1.4 CANCER[15-20]

Clinically, cancer is the name given to a large family of diseases, may be a hundred or more, which vary in age of onset, rate of growth, state of growth, state of cellular differentiation, diagnostic delectability, invasiveness, metastasis potential and response to treatment and prognosis. Cancer occurs when cells become abnormal and keep dividing and forming new cells. Normally, cells divide to form new cells only when the body needs them. If cells divide when new ones are not needed, they form a mass of excess tissue, called a tumor. Tumors can be benign (not cancer) or malignant (cancer).

The cells in the malignant tumors can damage and invade nearby tissues and organs. Cancer cells can also break away from the malignant tumor and travel through the bloodstream to form new tumors in other parts of the body. The spread of cancer is called metastasis. Over 1 million cases of cancer occur in the United States every year, not including basal cell and squamous cell skin cancers, which add another 700,000 cases annually. Although it is considered as a disease of aging, cancer can occur at any time. On average, the diagnosis of the most common types of cancer comes at about age 67. Although cancer is relatively rare in children, it is still a leading cause of death between ages 1 and 14. Millions of people alive today have had some type of cancer. Of these, about half are considered cured. The good news is that more and more people are now being cured of their cancers. This progress

is due to better techniques of diagnosis and treatment.[15]

- **Causes**[15]

In many cases, the causes of cancer are not clear, but both external and internal factors play a role. Cigarette smoking is a major causal factor. Other than that, diet, genetic mutation, exposure to UV light and carcinogenic chemicals may also cause cancer.

- **Prevention**[15]

The best way to reduce deaths from cancer is to prevent it. Medical doctors generally agree that about one-third of all human cancers are directly related to cigarette smoking. For smokers, the risk of cancer is much higher than that of the nonsmokers. Excluding the UV rays of sunlight which cause skin cancer, the next most common cited cancer-causing factor is diet. The National Cancer Institute and the American Cancer Society recommend a diet low in fat, high in natural fiber, and rich in fruits and vegetables. Chemoprevention on the other hand is simply prevention with drugs. The word “drugs” is used to include dietary supplements, hormones, and vitamins etc., as well as real drugs such as aspirin and other synthetic agents used for therapeutic purposes. The number of chemo preventive agents is increasing.

- **Treatments**[15]

Surgery is the oldest and still the most common treatment for cancer. Radiation therapy is the use of ionizing radiation to treat cancer. Ionizing radiation can be delivered using photon beams and particle beams. Radiation therapy is used at some point in the treatment of more than half of all cancer cases. High-energy X-rays are used to damage cancer cells and stop them from growing and spreading.

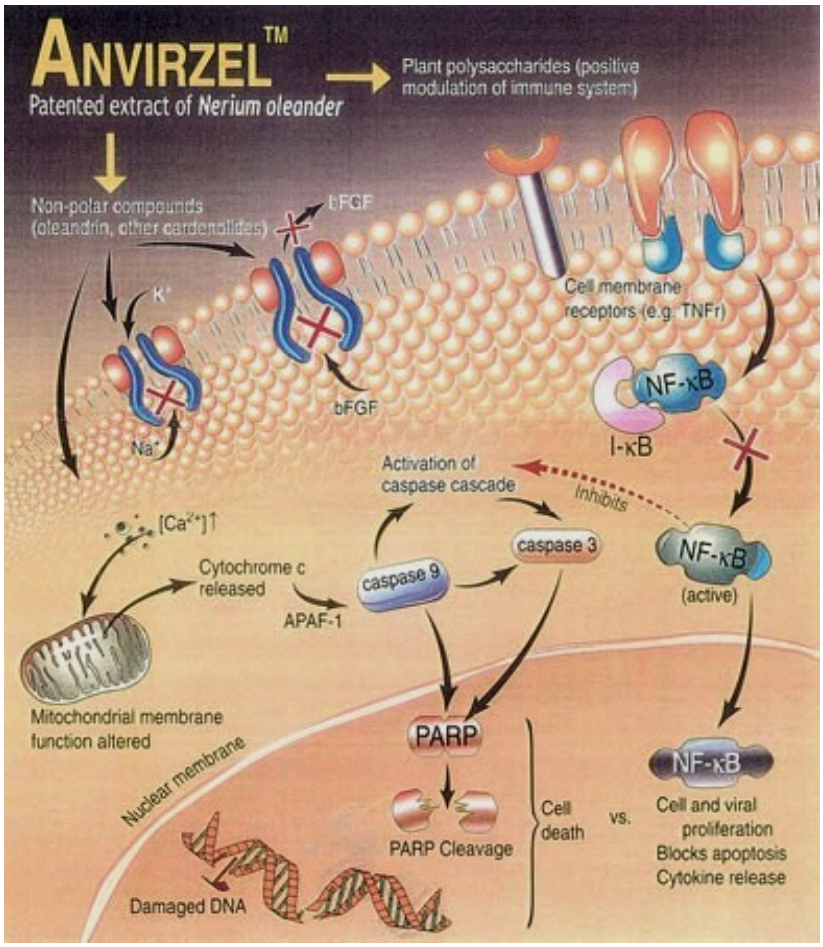
It can be used to shrink a tumor before surgery, but it is often used after surgery. Like surgery it is a local treatment; it affects the cells only in the treated area. Hormone therapy is used to keep cancer cells from getting the hormones they need to grow. It is often used as a follow-up to surgery. Reconstructive surgery is when one part of the body is replaced with another part. Chemotherapy is the use of drugs to kill cancer cells. Unlike surgery and radiation therapy it is systemic; it works throughout the body. A single drug or a combination of drugs may be used. Chemotherapy is often used after surgery to kill any hidden cancer cells that remain in the body.

- **Natural products in cancer chemotherapy**[16-20]

Drugs from plants (natural product drugs) have played a dominant role in pharmaceutical care for the treatment of various diseases, especially cancer. In addition to its use to treat heart failure, *Nerium oleander;* Apocynaceae is also known to be toxic against a wide range of tumor cells. The pentacyclic triterpenoids isolated from *Nerium oleander;* possess cytotoxic activity. Anvirzel™ is an aqueous extract of the plant *Nerium oleander* which has been utilized to treat patients with advanced malignancies. Teniposide a chemical analog of the natural product podophyllotoxin shows activity against Hodgkin's disease and other malignant lymphomas, pediatric refractory neuroblastoma, and brain tumors in adults. The alkaloid Camptothecin was first isolated from the tree *Camptotheca acuminate.* It has good activity against various cancers in the laboratory, but is too insoluble for clinical use. Various water-soluble analogs of Camptothecin (e.g. topotecan) have been developed, however, and have found significant clinical use. Some other plant species which are used in cancer therapies are *Maytenus buchananii, Triptigium wilfordii, Cephalotaxus harringtonia.* The glycorandomization of cardiac glycoside leads to analogs that display significantly enhanced potency and tumor specificity when a universal chemical glycosylation method that employs reducing sugars and requires no protection or activation is used. This suggests a divergent mechanistic relationship between cardiac glycoside induced cytotoxicity and Na^+/K^+-ATPase inhibition. There is a potential of chemical

modification to reduce the cardiovascular side-effects and improve the anti-cancer activity of new molecules. Ouabain and other cardiotonic steroids kill renal epithelial and vascular endothelial cells via their interaction with the Na^+/K^+-ATPase-subunit, but independently of elevation of the $[Na^+]_i/[K^+]_i$ ratio.

Figure 1.4: Mechanism of action of Anvirzel™



1.5 ENDOPHYTIC FUNGI[21-32]

Microorganisms have long served mankind by virtue of the myriad of the enzymes and secondary compounds that they produce. Furthermore, only a relatively small number of microbes are used directly in industrial applications (e.g. cheese, wine and beer making), in environmental clean-up operations and in the biological control of pests and pathogens. It seems that we have by no means

exhausted the world of its hidden microbes, and a much more comprehensive search of the Earth's various niches might yet reveal novel microbes which have direct usefulness to human societies. These uses could be either of the microbes itself, or one or more of its natural products.

The diversity of microbial life is enormous and the niches in which microbes live are truly amazing, ranging from deep ocean sediments to the Earth's thermal pools. The diversity of macroorganisms is probably mirrored by the diversity of microorganisms. That is, areas of the Earth inhabited by an enormous diversity of higher life forms are probably also inhabited by a high diversity of microorganisms. One specialized and unique biological niche that supports the growth of microbes is the intracellular space between cells of higher plants. It turns out that each plant supports a suite of microorganisms known as endophytes. These microorganisms do not cause overt symptoms on the plants in which they live. Furthermore, because so little work on these endophytes has been done, it is suspected that untold numbers of novel fungal and bacterial genera exist as plant-associated microbes and their diversity might parallel that of the higher plants. Such a specific search is governed by the fact that some endophytes could have coevolved with their respective higher plant and, as a result, already exist compatibly with a higher life-form. Thus, we have begun a concerted search for novel endophytic microbes, with the prospect that they could produce novel bioactive products as well as possess processes that might prove useful.

It is obvious that any discovery of novel microbes will have implications in virtually all of the standard processes of industrial microbiology because scale-up fermentation of the microbe will be necessary.[21]

A variety of animals and plants bear symbiotic microorganisms that contribute to their nutrition or defense against predators and parasites. These relationships are widely considered as mutualistic, i.e. both the animal/plant host and the microbial symbiont from the association. By definition, an endophytic fungus lives in mycelial form in biological association with the living plant, at least for some time. Therefore the minimal requirement before a fungus is termed an 'endophyte' should be the

demonstration of its hypha in living tissue. Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds. Further, endophytic fungi may also produce metabolites with thermo protective role.[22-24]

- **Isolation of Endophytic Fungi**

It is important to establish a specific protocol for the isolation of endophytes from a given plant, particularly as 90-99% of microorganisms are not readily cultivable. The most frequently isolated microorganisms are endophytic fungi. Scrutiny of the literature shows that there is little difference in the frequency of success of various isolation protocols, and that standardized procedures inevitably fall to result in the isolation of certain special endophyte(s).[25]

- **Methods for the Isolation of Endophytes**

The method most frequently utilized method to detect and quantify endophytic fungi involves isolation from surface-sterilized host plant tissue. For reports profiling inventors of endophyte species occurrence and diversity, this is currently the most practiced approach, although fungal biologists recognize that some endophyte groups may be undetected or under-represented, and in particulate, isolated that are unable to sporulate in culture may need to be evaluated by other means, host species, sampling strategy, host-endophyte and inter-endophyte interactions, tissue types and ages, geographic and habitat distributions, types of fungal colonization, culture conditions, surface sterilants, and selective media all influence the detection and enumeration of endophytic fungi, techniques used for isolation, maintenance, identification, and preservation of all endophytic fungus.

Detailed practical information on methods for the isolation of filamentous fungi from various substrata, including techniques, selective agents, and the most common media, is available in the literature. Surface sterilization of plant material is usually accomplished by treatment with a strong oxidant or general disinfectant for a period, followed by a sterile rinse. Household bleach (NaOCl), usually diluted in water to concentrations of 2-10% is the most commonly used surface sterilants.

Similarly effective oxidants include 3%H_2O_2 and 2%$KMnO_4$. Furthermore, the efficacy of surface sterilization can be substantially improved by combining with a wetting agent. This is particularly appropriate for hydrophobic or densely pubescent leaves. Ethanol (70-95%) is the most commonly used wetting agent in this respect, however, it has limited antibiotic activity, and thus is not used alone as a surface disinfectant. Other surfactants such as tween 80 have found use as wetting agents to enhance surface sterilization of the host plants.[25]

- **Isolation Procedures**

The host plant should be unambiguously identified in any endophyte-related study, and its global position, as defined by location, latitude, longitude and altitude, should be recorded. Endophytes are generally isolated after cutting individual plant organs into segments (3-5 mm long) followed immediately by treatment with bleach. Alternatively, plant material is surface treated with 70% ethanol and then dried under a laminar flow hood, two to three tissue segments are removed every 2-3 minutes and vigorously rinsed in sterile distilled water.

These pieces then are pressed into potato dextrose agar (PDA), and the plates are incubated at room temperature for 3-4 weeks. Rapidly growing fungi that appear within the first two weeks are generally discarded since they are most probably contaminants. After 2-4 weeks, white to off- white colonies of endophytes become visible.

Plates are prepared in triplicate to eliminate the possibility of contamination or heterokaryosis. It is advisable to remove outer tissues with a sterile knife blade, and the newly formed surfaces are placed carefully onto agar plates or PDA medium co-supplemented with 200 µg/ml ampicillin and 200 µg/ml streptomycin to suppress bacterial growth until the mycelium or colony originating from the segments appears. After several days of incubation, hyphal tips of the fungal endophyte are removed and transferred to newly prepared PDA plates. Some bacterial species such as *streptomyces* spp. can survive this treatment. For identification purposes, the endophytes are trained to sporulate on pre-

treated plant materials.

In order to isolate endophytes from plant seeds, the deglume is required to be removed together with contaminants associated with the dry glumes. This is achieved by rubbing the seeds vigorously between the hands and then rinsing the seeds for 15-20 minutes with a bleach solution. The isolation of endophytic bacteria is often accomplished by pasting onto nutrient agar plates the trituration of plant tissues surface-disinfected with various disinfectants such as sodium hypochlorite, ethanol, hydrogen peroxide, mercuric chloride, or a combination of two or more of these.[25, 26]

- **Identification**

Rigorous identification of endophytes requires microscopic examination of the host tissue and relies to a significant extent on the taxonomic expertise of the examiner. Morphological examination is performed by scrutinizing the culture, the mechanism of spore production, and the characteristics of the spores. This is especially valuable for isolates failing to produce spores or identifiable structures. Sometimes, optimization of growth conditions aiming at inducing sporulation of endophytes is a trial-and-error process. Each of the isolated fungal strains is separately inoculated on PDA, CMA, CA, WSA and PCA media in Petri dishes to achieve optimum conditions for sporulation. Moreover, endophytic fungi that neither grow nor sporulate in culture can only be detected and identified by other means such as a comparison of ribosomal DNA (rDNA) gene sequences, an analysis that can be used to determine phylogenic relationships.

Accordingly, endophytic isolates are often identified using a combination of morphological and molecular methods. Special caution has to be taken when closely related or morphologically similar endophytic fungi are under identification. The morphological features of some fungi are usually medium dependent, and some cultural conditions can affect substantially vegetative and sexual incompatibility. Thus, the morphological character of endophytes should be coupled with the available molecular evidence to enable significant differentiation between closely related species. For newly

discovered endophytic fungi, morphology-based identification is confirmed by 18S rDNA sequence comparisons or internal transcribed spacer (ITS1 and ITS2) and 5.8S rDNA sequence examinations. An endophytic isolate from the roots of vascular plants was characterized on the basis of cultural and morphological properties and PCR/RFLP analysis of the ITS region and a portion of the 28S subunit of rDNA.[27]

- **Endophytes: a rich source of functional metabolites**

In the continual search by both pharmaceutical and agricultural industries for new products, natural selection has been found to be superior to combinatorial chemistry for discovering novel substances that have the potential to be developed into new industrial products. Since natural products are adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes. Endophytic fungi inhabit such a biotope. In the course of the last 12 years, 6500 endophytic fungi were isolated from herbaceous plants and trees, screened for biological activities, and isolated and determined the structures of the biologically active compounds.

The substances isolated originated from different biosynthetic pathways: isoprenoid, polyketide, amino acid derivatives, and belonged to diverse structural groups: terpenoids, steroids, xanthones, chinones, phenols, isocumarines, benzopyranones, tetralones, cytochalasines, and enniatines. The potential role of the endophyte and its biologically active metabolites in its association with its host has been investigated. The concentrations of some plant defense metabolites are lower than in the control when the host is infected with a pathogen than with an endophyte.

The number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganism class. This may of course be a consequence of the high frequency of isolation of fungal endophytes from plants. Natural products from fungal endophytes have a broad spectrum of biological activity, and they can be grouped into several categories, including alkaloids,

steroids, terpenoids, isocoumarins, quinones, phenylpropanoids and lignans, phenol and phenolic acids, aliphatic metabolites, lactones, etc.[25, 28]

- **The Global Importance of Endophytic Fungus Derived Drugs**

Endophytes are the source of a broad range of bioactive metabolites, such as, antibiotics, antiviral, anticancer, antioxidant, insecticidal, antidiabetic, immunosuppressive compounds. There is a high diversity, spatial structure, and host affinity among foliar endophytes associated with a tropical tree (*Theobroma cacao*, Malvaceae) across lowland Panama. The inoculation of endophyte-free leaves with endophytes isolated frequently from naturally infected, asymptomatic hosts significantly decreases both leaf necrosis and leaf mortality when *T. cacao* seedlings are challenged with a major pathogen (*Phytophthora* sp.). In contrast to reports of fungal inoculation inducing systemic defense, it was found that protection was primarily localized to endophyte-infected tissues. Further, endophyte-mediated protection was greater in mature leaves, which bear less intrinsic defense against fungal pathogens than do young leaves. *In vitro* studies suggest that host affinity is mediated by leaf chemistry, and that protection may be mediated by direct interactions of endophytes with foliar pathogens. Together, these data demonstrate the capacity of diverse, horizontally transmitted endophytes of woody angiosperms to play an important but previously unappreciated role in host defense.[29, 30]

Endophytes isolated from rice (*Oryza sativa*) used as the test plant produced two types of interactions; biofilms (bacteria attached to mycelia) and mixed cultures with no such attachments.[31]

Fungal endophytes were isolated from roots and leaves of epiphytic and lithophytic orchids in the genus *Lepanthes*; seven species, from rainforests in Puerto Rico, were sampled. The endophytes observed most frequently were *Xylaria* species and *Rhizoctonia*-like fungi, found in 29%of roots and 19%of leaves, and 45%of roots and 31%of leaves, respectively. Five deuteromycete genera were also isolated, occurring in 19%of roots and 43%of leaves (combined). At least nine species of *Xylaria* were found, with several species sometimes occurring in a single plant.[32]

2.1 GENERAL INFORMATION

Figure 2.1 Flowering Twing of *Nerium oleander*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Gentianales
Family	Apocynaceae (dogbane)
Genus	***Nerium*** L.
Species	***N. oleander***
Scientific name	*Nerium Oleander* Linn.
Common Names	Oleander, adelfa, laurier rose, rosa laurel, rose bay, rosa francesa.[33]

- **Origin and distribution**

Nerium oleander Linn distributed in the Mediterranian region and sub-tropical Asia is indigenous to the Indo-Pakistan subcontinent. It is a native to a broad area from Morocco and Portugal eastward through the Mediterranean region and southern Asia to Yunnan in southern parts of China. The plant is also found commonly in the southern United States and most of California.[33, 34]

Form	Large shrub can be trained to single or multi stemmed tree
Seasonality	evergreen
Size	large variation with cultivars; heights from 3 to 20ft, spreads from 3-12ft
Leaves	simple, linear, 3-12in long, whorled around stem, glossy dark green on top and lighter underneath with prominent mid-rib
Flowers	Terminal clusters, may be yellow, white, pink or red; may be single or double; bloom in spring and throughout summer
Fruit	3-5in long pods, green ripening to brown, splits to release airborne seed
Stems/Trunks	gray, extremely tough, can be trained or pruned as desired
Range/Origin	Mediterranean and Asia
Hardiness	varies with variety, standard types to low teens, dwarf varieties more tender; damage seen on dwarfs in high teens; all types recover quickly from frost damage[33, 34]

2.2 CULTIRAL REQUIREMENTS[33]

Exposure	full sun to part shade, takes reflected heat
Water	drought tolerant; supplement during hot season,
Soil	tolerant, good drainage
Propagation	vegetative cuttings
Maintenance	low to moderate; pruning to control size or shape

2.3 FOLKORIC USES

- Herpes zoster (skin): Crush leaves, mix oil and apply on lesions. Do not apply on raw surface. Milky juice of the plant is irritating. Caution: Not to be taken internally.
- Herpes simplex: Mix 1 cup of chopped leaves and bark with 2 tablespoons of oil. Apply to lesions 3 times daily.
- Ringworm: Chop a foot long branch and mix with 1 cup chopped fresh young leaves. Mix the juice with 5 drops of fresh coconut oil. Apply 3 times daily.
- Snake bites: Pound 10 leaves and a piece of branch. Apply poultice to the wound.
- Roots, locally and internally, used for abortion.
- Roots, made into paste with water, used for hemorrhoids.

- Roots and bark externally for eczema, snake bites and as insecticide.
- Fresh leaves applied to tumors to hasten suppuration.[33]

2.4 CHEMICAL CONSTITUENTS

Nerium oleander's leaves contain two principles: neriin and oleandrin, glucosides with properties similar to digitalin. The seeds contain phytosterin and l-strophnathin. The bark contains toxic glycosides: rosaginin and neriin, volatile oil, fixed oil. Various parts of the plant are known to yield cardenolides, pregnanes and triterpenes. The evidence of isolation of two cytotoxic pentacyclic triterpenoids, cardenolide (3b-hydroxy-5b-carda-8, 14, 16, 20(22)-tetraenolide) and four CNS depressant cardenolides including a new cardenolide, neridiginoside and three known constituents, nerizoside, neritaloside and odoroside-H, which exhibited CNS depressant activity in mice from the leaves of *N. oleander*, is found in literature. Kaneroside and neriumoside have been isolated from the fresh, undried, winter leaves of Nerium oleander. Polar glycosides gentiobiosyl-nerigoside and gentiobiosylbeaumontoside are isolated along with the major trioside, gentiobiosyl-oleandrin from air dried *Nerium oleander* leaves. Neritaloside, oleandrigenin, odoroside, and oleandrin are detected by HPLC/MS/MS from a novel oleander extract. Oleandrin production can be enhanced by tissue culture technique.[16, 34-39]

2.5 MEDICINAL USES

(Cancer; Cardiotonic; Diaphoretic; Diuretic; Emetic; Expectorant; Resolvent; Skin; Sternutatory)

The leaves and the flowers are cardiotonic, diaphoretic, diuretic, emetic, expectorant and sternutatory. The crude ethanolic extract of *Nerium oleander* possesses cardiotonic effect in the isolated

guinea pig hearts. A decoction of the leaves has been applied externally in the treatment of scabies, and to reduce swellings. This is a very poisonous plant, containing a powerful cardiac toxin, and should only be used with extreme caution. The root is powerful resolvent. Because of its poisonous nature it is only used externally. It is beaten into a paste with water and applied to cancers and ulcers on the penis. Oil prepared from the root bark is used in the treatment of leprosy and skin diseases of a scaly nature. The whole plant is said to have anticancer properties.

Anvirzel™ is an aqueous extract of the plant *Nerium oleander* which has been utilized to treat patients with advanced malignancies. Topical application of oleandrin possesses anti-tumor promoting effects. Oleandrin suppresses activation of nuclear transcription factor-κB and activator protein-1 and potentiate apoptosis induced by ceramide.

Treatment of Streptozotocin-Induced diabetic rats with glimepiride and *Nerium oleander* extract offered protection in terms of lipid profile, growth rate and renal function, indicating their antidiabetic potential.

Extract obtained from *Nerium oleander* has in many cases significantly prolonged life of patients with metastatic cancer disease.[40-44]

2.6 OTHER USES

(Dye; Hedge; Insecticide; Latex; Parasiticide; Soil stabilization)

The plant is used as a rat poison, a parasiticide and an insecticide. The pounded leaves and bark are used as an insecticide. A green dye is obtained from the flowers. The plant extracts is used as a wood preservative due to its antifungal properties.[45]

2.7 TOXICITY

Oleandrin ingested in small quantities (0.005% animal weight in horse) cause death. Oleander bark contains rosaginin which is known for its strychnine-like effect. The entire plant including the

milky white sap is toxic and any part can cause an adverse reaction. Oleander holds its toxicity even after drying. The literature review indicates that small children and domestic livestock are at increased risk of oleander poisoning. According to Toxic Exposure Surveillance System (TEES) in 2002 there were 847 known human poisonings in the United States related to oleander. There are innumerable reported suicidal cases of consuming mashed oleander seeds in South India. In animals, 0.5 mg/kg of body weight is lethal to many animals.

In cases of digitalis-like poisoning with suspicion of oleander ingestion, a combination of digoxin immunoassays may be useful to effectively rule out the presence of oleander.[31, 46]

1. LITERATURE REVIEW

1. **John P. Reeves et. al., (1979)[48]** isolated membrane vesicles from rabbit ventricular tissue rapidly accumulated Ca^{2+} when an outwardly directed Na^{+} gradient was formed across the vesicle membrane. Vesicles loaded internally with K^{+} showed only 10% of the Ca^{2+} uptake activity observed with Na+-loaded vesicles. Na^{+}-dependent uptake and efflux of Ca^{2+} were both inhibited by La^{3+}. The results indicate that cardiac membrane vesicles exhibit Na^{+}-Ca^{2+} exchange activity. Fractionation of the vesicles by density gradient centrifugation revealed a close correspondence between Na^{+}-Ca^{2+} exchange activity and specific ouabain-binding activity among the various fractions. This relationship suggests that the observed Na^{+}-Ca^{2+} exchange activity derives from the sarcolemmal membranes within the vesicle preparation.

2. **B. Chiarlo et. al., (1978)[55]** evaluated the oleandrin content of leaves of Nerium oleander L. monthly, over a period of 15 months, by a spectrophotometric method after solvent extraction, chromatographic separation on silica gel preparative/avers and column purification. The amount of oleandrin is connected with the cambium meristem rhythm of the stem during different months of the year. It has been shown that the increase of oleandrin content is connected with the cambium reactivation which, in turn, is affected by the weather conditions.

3. **Bina S. Siddiqui et. al., (1987)[36]** isolated two new cardiac glycosidcs, kaneroside and ncriumoside, from the fresh, undried, winter leaves of *Nerium oleander* and their structures established as 3~-O-(D-diginosyl~Zz-hydroxy-8.14~3poxy-S~car&-16: 17,20:22dicnolide and 3/3-O-(Ddiginosyl)-2x,l4/3dihydroxy-Sgcard-l6: l7,20:22dicnolide, respectively, through chemical and spectral studies.

4. **Naci M. Bor et. al., (1988)[44]** claimed that the extract obtained from Nerium Oleander has significantly prolonged life of patients with metastatic cancer disease where regression has been observed in some cases and total cure in others. Immunomodulator function observed by Roemer may explain the improvement recorded in many of them. These reports, however, have been opposed by others who maintain that besides the extract being toxic the evidence in favor of Nerium Oleander's benefical influence on cancer is insufficient. Still others have collected evidence indicating that it is in proposed doses nontoxic.

5. **Fumiko Abe et. al., (1992)[37]** isolated gentiobiosyl-nerigoside and gentiobiosyl beaumontoside along with the major trioside, gentiobiosyl-oleandrin from the air-dried leaves of *Nerium oleander*. Minor triosides also include glycosides of 8/l-hydroxy- and A16-8fi-hydroxy-digitoxigenin, and Ai6 -neriagenin, along with glycosides of known cardenolides, oleandrigenin, digitoxigenin, adynerigenin, neriagenin and their A16-derivatives.

6. **Bina S. Siddiqui et. al., (1995)[16]** described the isolation and structure elucidation of two novel cytotoxic pentacyclic triterpenoids cis-karenin *(3fl-hydroxy-28-Z-p-coumaroyloxy-urs-12-en-27-oic* acid) and *trans-karenin (3-fl-hydroxy-28-E-p-coumaroyloxy*urs-12-en-27-oic acid) from the leaves of *Nerium oleander.*
7. **Roland Valdes, Jr. et. al., (1996)[47]** have investigated the cross-reactivities of oleandrin and its aglycone metabolite, oleandrigenin, in several commercially available digoxin immunoassays; assessed their ability to inhibit Na,K-ATPase catalytic activity; and measured their binding to proteins in serum. As assayed with ACS: 180, Stratus, RIA, On-Line, and TDx digoxin assays, oleandrin at 100 Lmol/L in digoxin-free serum gave apparent digoxin values of 0, 0.83, 2.24, 2.37, and 5.34 nmolfL, respectively, whereas oleandrigenin at that concentration gave results of 0, *0.52,* 0.77,4.94, and 1.40 nmol/L. Study of Na,K-ATPase inhibition showed IC50 values (Lmol/L) of

0.22 for ouabain, 0.62 for oleandrin, 1.23 for oleandrigenin, and 2.69 for digoxin. At *25*{17966}%C, of oleandrin and 48% of oleandrigenin were bound to serum proteins. In cases of digitalis-like poisoning with suspicion of oleander ingestion, a combination of digoxin immunoassays may be useful to effectively rule out the presence of oleander.

8. **C.M. Hasan et. al., (1999)**[35] isolated a new cardenolide, 12b-hydroxy-5b-carda-8,14,16,20(22)-tetraenolide (2) from the roots of *Nerium oleander*. Biological screening of the compound revealed antibacterial and digoxin-like cardiac activities.

9. **Bina S. Siddiqui et. al., (1999)**[34] studied the central nervous system (CNS) depressant effect in mice of a bioactivity directed isolation of the methanolic extract of the fresh, uncrushed leaves of *Nerium oleander*. As a result, four CNS depressant cardenolides including a new cardenolide, neridiginoside and three known constituents, nerizoside, neritaloside and odoroside-H, have been isolated which exhibited CNS depressant activity in mice at a dose of 25 mg/kg. The structure of neridiginoside was elucidated as 3b-O- (D-diginosyl)-5b,14b-dihydroxy-card-20(22)-enolide, using spectroscopic methods including one-dimensional and two-dimensional NMR (COSY-45, NOESY, J-resolved, HMQC and HMBC). The known compounds have been identified through spectral studies and comparison of data with those reported in the literature.

10. **Xiaomin Wang et. al., (2000)**[38] have developed an HPLC/MS/MS method for the characterization and quantification of the cardiac glycosides oleandrin, odoroside, neritaloside and the aglycone oleandrigenin, all contained in a patented-hot-water extract of *Nerium oleander* L (Anvirzel), using a hybrid tandem quadrupole time-of-flight (QqTOF) mass spectrometer. Collision-induced dissociation (CID) mass spectra of oleandrin, oleandrigenin, odoroside, and neritaloside were obtained with greater than 5 ppm mass accuracy and resolution routinely in excess of 8000 (fwhm).

The detection limit for oleandrin of 20 pg (injected) was realized when the precursor-to-product ion transition, *m*/*z* 577 f 373, was monitored. They have also applied the analytical method to the determination of oleandrin, oleandrigenin, neritaloside, and odoroside in human plasma following an intramuscular injection of Anvirzel.

11. **Christopher M. M. Franco et. al., (2003)**[52] isolated filamentous actinobacteria from surface-sterilized root tissues of healthy wheat plants (*Triticum aestivum* L.). Roots were surface-sterilized by using ethanol and sodium hypochlorite prior to the isolation of the actinobacteria. Forty-nine of these isolates were identified by using 16S ribosomal DNA (rDNA) sequencing and found to belong to a small group of actinobacterial genera including *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardiodes* spp. In particular, several isolates exhibited high 16S rDNA gene sequence homology to *Streptomyces caviscabies* and *S. setonii*. None of these isolates, nor the *S. caviscabies* and *S. setonii* type strains, were found to carry the *nec1* pathogenicity-associated gene or to produce the toxin thaxtomin, indicating that they were nonpathogenic. These isolates were recovered from healthy plants over a range of geographically and temporally isolated sampling events and constitute an important plant-microbe interaction.

12. **R. O. Adome et. al., (2003)**[40] have tested the crude ethanolic extracts of the dried leaves of *Nerium Oleander* against the following parameters in the isolated guinea pig hearts: force of contraction, heart rate and cardiac flow. The extracts brought about dose-dependent increases in all these parameters from their baseline readings. Compared with graded doses of digoxin the effects closely mirrored the activities in a dose dependent manner. At the mechanism of action level, it would appear the extract works in the same as digoxin since their dose-contraction reponse curves are parallel. This finding would tend to provide a strong rationale for the herb's traditional use in cardiovascular illness.

13. **R.X. Tan et. al., (2004)**[50] recognized *Aspergillus fumigatus* CY018 as an endophytic fungus for the first time in the leaf of *Cynodon dactylon*. By bioassay-guided fractionation, the EtOAc extract of a solid-matrix steady culture of this fungus afforded two new metabolites, named asperfumoid (**1**) and asperfumin (**2**), together with six known bioactive compounds including monomethylsulochrin, fumigaclavine C, fumitremorgin C, physcion, helvolic acid and 5,8-epidioxy-ergosta-6,22-diene-3-ol as well as other four known compounds ergosta-4,22-diene-3-ol, ergosterol, *cyclo*(Ala-Leu) and *cyclo*(Ala-Ile). Through detailed spectroscopic analyses including HRESI-MS, homo- and hetero-nuclear correlation NMR experiments (HMQC, COSY, NOESY and HMBC), the structures of asperfumoid and asperfumin were established to be spiro-(3-hydroxyl-2,6-dimethoxyl-2,5-diene- 4-cyclohexone-(1,3)-5-methoxyl-7-methyl-(1H, 2H, 4H)-quinoline-2,4-dione) and 5-hydroxyl-2-(6-hydroxyl-2-methoxyl-4-methylbenzoyl)-3,6-dimethoxyl-benzoic methyl ester, respectively. As a result, asperfumoid, fumigaclavine C, fumitremorgin C, physcion and helvolic acid were shown to inhibit *C. albicans* with MICs of 75.0, 31.5, 62.5, 125.0 and 31.5 g/mL, respectively.

14. **R. Ananthan et. al., (2004)**[60] investigated the effect of *Gymnema montanum* leaf extract (GLEt) on tissue antioxidant defense systems in alloxan-induced diabetes in rats. GLEt was administered orally at doses of 50, 100, and 200 mg/kg of body weight for 30 d, after which liver and kidney tissues were assayed for the degree of lipid peroxidation by means of markers, reduced glutathione content and activities of catalase, superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase. Treatment of diabetic rats with GLEt increased the antioxidant levels. Liver and kidney from diabetic animals exhibited disturbances in antioxidant defense when compared with normal rats. GLEt at a dose of 200 mg/kg of body weight exhibited a significant effect as compared with 50 and 100 mg/kg of body weight. These effects were compared with glibenclamide, a reference drug. They concluded that, in diabetes, liver and kidney tissues are more vulnerable to oxidative

stress and show increased lipid peroxidation. The antioxidant responsiveness mediated by *G. montanum* may be anticipated to have biological significance in eliminating reactive free radicals that may otherwise affect normal cell functioning and provide a scientific rationale for the use of *G. montanum* as an antidiabetic plant.

15. **Sergei N. Orlov et. al., (2005)[20]** reported that ouabain and other cardiotonic steroids (CTS) kill renal epithelial and vascular endothelial cells via their interaction with the Na^+/K^+-ATPase-subunit, but independently of elevation of the $[Na^+]i/[K^+]i$ ratio. They did not observe any effect of extra- (EGTA) and intracellular (BAPTA) Ca2+-chelators, [Ca2+]i-raising compounds (thapsigargin, ATP), inhibitors of Ras signaling (hydroxyfarnesylsulphosphoric acid), PI3K (wortmannin), MAPK ERK1/2 kinase (PD98059), tyrosine kinases (genistein) as well as activators (4-PMA,8-Br-cAMP, 8-Br-cGMP, forskolin) and inhibitors (calphostin) of serine–threonine kinases on MTT staining and death of ouabain-treated cells. Ouabain did not affect cellular redox state and the production of superoxide anion and hydroperoxide. Their results show that none of the above-listed signaling systems plays a major role in the development of Na^+i ,K^+_i -independent death machinery triggered by CTS interaction with the Na^+,K^+-ATPase-subunit.

16. **Jerry B Lingrel et. al., (2005)[9]** showed that binding site for cardiac glycosides in Na^+/K^+-ATPase plays an important role in the regulation of blood pressure, and it specifically mediates adrenocorticotropic hormone (ACTH)-induced hypertension in mice. They used genetically engineered mice in which the Na^+/K^+-ATPase 2 isoform, which is normally sensitive to cardiac glycosides, was made resistant to these compounds. Chronic administration of ACTH caused hypertension in WT mice but not in mice with an ouabain-resistant 2 isoform of Na^+/K^+-ATPase. Their results demonstrate that the cardiac glycoside binding site of the isoforms of the Na^+/K^+-ATPase have a physiological function and supports the hypothesis for a role of the endogenous

cardiac glycosides.

17. **William J. Welsh et. al., (2005)[8]** proposed a binding mode for digoxin and several analogues to the Na^+/K^+-ATPase. A 3D-structural model of the extracellular loop regions of the catalytic a1-subunit of the digitalis-sensitive sheep Na^+/K^+-ATPase was constructed from the crystal structure of an $E1Ca^{2+}$ conformation of the SERCA1a and a consensus orientation for digitalis binding was inferred from the in silico docking of a series of steroid-based cardiotonic compounds. Analyses of species-specific enzyme affinities for ouabain were also used to validate the model and, for the first time, propose a detailed model of the digitalis binding site.

18. **Tarek Mekhail et. al., (2006)[17]** reported a phase 1 trial to determine the maximum tolerated dose (MTD) and safety of Anvirzel™ in patients with advanced, refractory solid. Patients were randomized to receive this agent by intramuscular injection at doses of 0.1, 0.2, 0.4 ml/m2/day with subsequent patients receiving 0.8 or 1.2 ml/m2/day sequentially. Eighteen patients were enrolled and completed at least one treatment cycle of three weeks. Most patients developed mild injection site pain (78%). Other toxicities included fatigue, nausea, and dyspnea. Traditional dose limiting toxicity was not seen, but the MTD was defined by injection volume as 0.8 ml/m2/day. No objective anti-tumor responses were seen. Anvirzel™ can be safely administered at doses up to 1.2 ml/m2/day, with the amount administered intramuscularly limited by volume. The recommended phase II dose level is 0.8 ml/m2/day.

19. **Katarina Popov-Pergal et. al., (2006)[61]** synthesized new functionally substituted 5-arylidene-2,4-dioxotetrahydro-1,3-thiazole-3-carboxylic acid cholesteryl esters from 2,4-dioxotetrahydro-1,3-thiazole and evaluated for their *in vitro* cytotoxicity against several human tumor cell lines and one normal lung fibroblast cell line.

20. **Maged M. Yassin et. al., (2007)**[43] studied the protective potential of glimepiride and Nerium oleander extract on lipid profile, body growth rate, and renal function in streptozotocin-induced diabetic rats. The animals were divided into control and experimental groups. The experimental group was rendered diabetic by intraperitoneal injection of a single dose of 50 mg/kg body weight streptozotocin. Rats with glucose levels >200 mg/dl were subdivided into 3 sub-groups. Rats in the first sub-group remained without treatment and were considered diabetic. Those in the second and third subgroups were orally administered 0.1 mg/kg body weight daily glimepiride and 250 mg/kg body weight daily Nerium oleander extract, respectively, for 4 weeks. In the streptozotocin induced diabetic rats, serum triglycerides and cholesterol were significantly increased whereas body growth rate was markedly decreased compared to the controls. In contrast to uric acid and creatinine, urea concentrations were markedly elevated. Treatment of diabetic rats with glimepiride or plant extract improved all of these parameters, indicating their antidiabetic efficacy.

21. **Gary Strobel et. al., (2007)**[51] isolated a novel endophytic fungal genus *Muscodor* that produces bioactive volatile organic compounds (VOCs). This fungus, as well as its VOCs, has enormous potential for uses in agriculture, industry and medicine. *Muscodor albus* produces a mixture of VOCs that act synergistically to kill a wide variety of plant and human pathogenic fungi and bacteria. This mixture of gases consists primarily of various alcohols, acids, esters, ketones and lipids. Artificial mixtures of the VOCs mimic the biological effects of the fungal VOCs when tested against a wide range of fungal and bacterial pathogens. Many practical applications for 'mycofumigation' by M. albus have been investigated and the fungus is now in the market place.

22. **Ramesh Chandra et. al., (2007)**[59] studied the contribution of oxidative stress from the standpoint of lipid and protein damage, alteration in endogenous antioxidant enzymes and effects of newly

synthesized compounds, 5-[4-2-(6,7-Dimethyl-1,2,3,4-tetrahydro-2-oxo-4-quinoxalinyl)ethoxy]phenyl]methylene]thiazolid- ine-2,4-dione, (C1) in normal/alloxan-induced diabetic rats. Its effect was compared to two well-known TZDs, namely pioglitazone and rosiglitazone. It has been concluded from results that after thirty days of administration of C1, Pg and Rg in alloxan-induced diabetic animal groups, the blood glucose level decreased, more remarkably in C1 treated group. Their results suggest that the newly synthesized TZD derivative (C1) has a potential to act as antihyperglycemic and antioxidant agent. In addition, for all parameters checked, it has better efficacy than rosiglitazone and is as effective as pioglitazone.

23. **Mostafa Mesbah et. al., (2007)**[39] established suspension cultures derived from *Agrobacterium tumefaciens*-transformed calli in *Nerium oleander* L. The presence of the bacterial T-DNA in the transformed calli was detected by the polymerase chain reaction as well as plant hormone autotrophy. The ability of the cultures to accumulate oleandrin was confirmed using high performance liquid chromatography. The oleandrin yield reached a maximum of 3.164 mg l−1 in 25-days upon employing *A. niger* elicitors. It was 8.8-fold higher than that of control cultures which reached a maximum of 0.35 mg l−1. All the transformed cultures were grown in hormone-autotrophic MS medium supplemented with 30 g l−1 sucrose at 25 ◦C under diffuse fluorescent light providing 37.5 mmol−2 s−1 light intensity in 12 h photoperiods.

24. **Wu-Yang Huang et. al., (2007)**[49] investigated fungal endophytes associated with a medicinal plant, *Nerium oleander* L.(Apocynaceae). A total of 42 endophytic fungal strains were isolated from the host plant. Total antioxidant capacity, xanthine oxidase inhibitory activity, antimicrobial activity, and total phenolic content (TPC) were evaluated for 16 representative fungal cultures grown in improved Czapek's broth and for the host plant. A significant positive correlation was found between antioxidant capacity and TPC in the tested samples. Most of the endophytic fungal

cultures tested have a wide range of antimicrobial activities, which were not very strong, but much better than those of the host plant. The major bioactive constituents of the fungal cultures were investigated using LC-ESI-MS and GC-MS, and preliminary identification detected phenolics (e.g. phenolic acids and their derivatives, flavonoids) and volatile and aliphatic compounds. This study shows that the endophytic fungi isolated from *N. Oleander* can be a potential antioxidant resource.

25. **Osman Goktas et. al., (2007)**[45] determined the antifungal properties of poisonous plant extracts from oleanders (*Nerium oleander* L.) when used as a wood preservative. The extract was prepared from oleanders leaves and flowers in 96% ethyl alcohol. The wood blocks of Turkish oriental beech (*Fagus orientalis* L.*)* and Scots pine (*Pinus sylvestris* L.) were impregnated with the extracts. The abilities of the extract to suppress attack by *Postia placenta* (Fr.) (brown rot) and *Trametes versicolor* (L: Fr.) Quel. (a white-rot) was investigated. Treated blocks were exposed to *P. placenta* and *T. versicolor* attacks for 12 weeks by following the soil-block method. While untreated wood specimens have weight loss ranging between 27.37 and 30.66% for *P. placenta* and 8.64 and 24.06% for *T. versicolor*, the wood treated with the extracts is of weight loss between 5.54 and 10.98% for *P. placenta*, and between 5.02 and 28.25% for *T. versicolor*. The lowest weight loss was found to be for beech wood (5.02%) impregnated with the extract of *oleander* at a concentration level of 0.25% against *T. versicolor.* While the highest weight loss was also on the beech wood (28.25%) treated with the same extract at the level of 0.50% concentration against *T. versicolor.* In conclusion, the extracts could be used as effective wood preservative.

26. **R.N. Kharwar et. al., (2007)**[51] studied the endophytes of *Azadirachta indica* A. Juss (the neem tree) growing in several of its natural habitats in India. A total of 233 isolates of endophytic fungi representing 18 fungal taxa were obtained from segments of bark, stem, and leaves of this tree. Hyphomycetes (62.2%) were the most prevalent followed by the Coelomycetes (27.4%) and

Mycelia Sterilia (7.7%). Endophytic colonization frequency was also greater in leaves (45.5%) than bark (31.5%). The dominant endophytic fungi isolated were *Phomopsis oblonga, Cladosporium cladosporioides*, *Pestalotiopsis* sp., *Trichoderma* sp, and *Aspergillus* sp. Genera such as *Periconia, Stenella*, and *Drechslera* are reported here for the first time as endophytes from this host plant. The rich and sizeable collection of endophytic fungi from this specific plant may represent a unique source of one or more of the interesting and useful bioactive compounds normally associated with *A. indica* such as the azadirachtins and related tetranortriterpenoids.

27. **Nageswara Rao et. al., (2007)**[57] examined the effect of methanolic extract of *Talinum portulacifolium* (METP) leaves for its anti-hyperglycemic activity against alloxan induced diabetes. The anti-hyperglycemic effect of METP was evaluated in alloxan induced diabetic rats by monitoring its effect on blood glucose, serum lipid profiles (triglycerides and total cholesterol), liver glycogen, malondialdehyde and reduced glutathione (GSH) levels. The rats, pretreated with METP (400 mg/kg p.o) improved oral glucose tolerance compare to glucose fed rats. After oral administration of METP in diabetic rats for 15 days, the blood glucose, lipid profile and malondialdehyde has significantly decreased, while liver glycogen and reduced glutathione were increased significantly. Histopathological studies of the pancreas of these animals showed comparable regeneration by METP, which were earlier necrosed by alloxan. Antihyperglycemic activity of METP was compared with oral hypoglycemic agent, glibenclamide.

28. **José Alberto Pereira et. al., (2008)**[56] studied the total phenols content and antioxidant and antimicrobial activities in walnut (Juglans regia L.) green husks aqueous extracts of five different cultivars (Franquette, Mayette, Marbot, Mellanaise and Parisienne). The antioxidant capacity of aqueous extracts was assessed through reducing power assay, scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and b-carotene linoleate model system. A concentration-

dependent antioxidative capacity was verified in reducing power and DPPH assays, with EC50 values lower than 1 mg/mL for all the tested extracts. All the extracts inhibited the growth of Gram positive bacteria, being *Staphylococcus aureus* the most susceptible one with MIC of 0.1 mg/mL for all the extracts. The results obtained indicate that walnut green husks may become important in the obtainment of a noticeable source of compounds with health protective potential and antimicrobial activity.

4. AIM AND OBJECTIVE

The significance of natural products in the drug discovery and development processes has been reported extensively. The importance of natural products as sources of innovative therapeutic agents can be illustrated by the drugs used in the control of infectious diseases, cancer, lipid disorders, immunomodulation and heart diseases. However, one crucial aspect to be considered for a successful natural product-based drug discovery program is the selection of the natural source to be studied. It is important to take into account that unexplored and/or underexplored sources of biological diversity are often related to novel chemical diversity. Endophytic fungi have been considered to be untapped sources of natural products and should be included in the search for new and innovative biologically active compounds.

The cost of cardiac glycosides of *Nerium Oleander* (oleandrin, oleandrigenin etc.) is very high (Rs.600000-1000000/gm). Average content of oleandrin is 0.018% weight/wet weight of leaf. An aqueous extract of the plant (Anvirzel™) is used to treat cancers. The endophytic fungi produce similar secondary metabolites or their derivatives as the host plant.

Evaluation of the biological activities of the endophytic fungi from *Nerium Oleander* is the main objective of the present study. The number of metabolites produced by fungal endophytes is larger than any other endophytic microorganism class. This may of course be a consequence of the high frequency of isolation of fungal endophytes from plant.

5. PLAN OF WORK

1. Collection and identification of host plant
2. Isolation of endophytic fungi
3. Primary screening
    - Giant colony technique
    - Baljet test
4. Cultivation of selected endophytic fungi on improved Czapek's broth medium
5. Separation and lyophilization of supernatant
6. Screening for the following biological activities:
    - Antifungal activity
    - Acute oral toxicity studies
    - Cardiotonic activity
    - Antidiabetic activity
    - Cytotoxic activity

6. MATERIALS AND INSTRUMENTS

TABLE 6.1 MATERIALS

Sr. No.	Name of Chemical	Supplier
1	Agar	HIMEDIA, MUMBAI
2	Alloxan tetrahydrate	SD FINE, MUMBAI
3	Ampicillin	HIMEDIA, MUMBAI
4	Carboxymethyl cellulose	LOBA CHEMICALS, MUMBAI
5	Copper sulphate	HIMEDIA, MUMBAI
6	Cycloheximide	HIMEDIA, MUMBAI
7	Diazepam	RANBAXY, MUMBAI
8	Dipotassium hydrogen phosphate	MERCK
9	Dulbecoo's phosphate buffer saline	MP BIOMEDICALS
10	EDTA	MP BIOMEDICALS
11	Ethanol	LOBA CHEMICALS, MUMBAI
12	Ferrous sulphate	HIMEDIA, MUMBAI
13	Fetal Bovine Serum	BIOCLOT
14	Gliclazide	CADILA, AHMEDABAD
15	Magnesium sulphate	NICE CHEMICALS, COCHIN
16	Methanol	LOBA CHEMICALS, MUMBAI
17	Orthophosphoric acid	QUALIGENS, MUMBAI
18	Phosphoric acid	SD FINE, MUMBAI
19	Picric acid	CENTRAL DRUG HOUSE, MUMBAI
20	Potassium chloride	MERCK
21	Rat chaw pallets	HINDUSTAN LEVER LTD, MUMBAI
22	Sabouraud dextrose agar	HIMEDIA, MUMBAI
23	Sodium bicarbonate	MP BIOMEDICALS
24	Sodium hydroxide pellets	NICE CHEMICALS, COCHIN
25	Sodium hypochlorite	NICE CHEMICALS, COCHIN
26	Sodium Nitrate	NICE CHEMICALS, COCHIN
27	Sodium-potassium tatarate	VIKASH PHARMA, MUMBAI
28	Streptomycin	NICHOLAS PIRAMAL, MUMBAI
29	Sucrose	SD FINE, MUMBAI
30	Sulfuric acid	SD FINE, MUMBAI
31	Trichloro acetic acid	MERCK
32	Trypsin	INVITROGEN
33	Vanillin	HIMEDIA, MUMBAI
34	Yeast extract	HIMEDIA, MUMBAI

6.2 INSTRUMENTS		
Sr. No.	**Name Of Instrument**	**Supplier**
1	Autoclave	New Lab
2	Biosafety cabinet classII	Esco
3	CO_2 incubator	RS Biotech, mini galaxy A
4	Colony counter	Neo Lab
5	Cooling centrifuge	Remi C24-BL
6	Deep freezer	Blue star
7	Double beam UV/VIS Spectrophotometer	ELICO, Mumbai
8	Electrocardiogram	BIOPAC MP30 system, Germany
9	Electronic Balance	Schimadzu, Japan
10	ELISA plate reader	Thermo
11	Fluorescence inverted microscope	Leica DM IL
12	Glass wares	Borosil
13	Glucometer	Roche
14	Incubator	Genuine
15	Laminar air flow	Scientec genuine
16	Micropipettes	Eppendorff
17	Refrigerator	Godrej
18	RO water system	Millipore
19	Rotary flash evaporator	Super fit, India ltd.
20	Shaker	Neo Lab
21	Sonicator	PCI, Mumbai
22	Vaccume Filteration unit	Super Fit, India
23	Water Bath	Genuine

7. EXPERIMENTAL INVESTIGATIONS

7.1 ISOLATION OF ENDOPHYTIC FUNGI[49-52]

- **Collection of Host Plant**

Fresh mature leaves, stems and roots of *Nerium oleander* Linn (Apocynaceae) were collected from a healthy plant from the local region of Tamilnadu. The plant was identified at Botanical survey of India (BSI) Coimbatore, India. The samples were taken to the laboratory and treated within 3 h.[49]

- **Isolation of endophytic fungi**

Samples of leaves, stems and root from *Nerium oleander* plants were first washed in running water. Washing included a sonication step to dislodge any soil and organic matter from the roots. The leaves were cut into segments (5 x 5 mm), and stems and roots were cut into pieces (10 mm in length). They were surface sterilized with 75% ethanol for 1 min and 2.5% sodium hypochlorite for 15 min, then rinsed in sterile water for three times and cut into 1 cm long segments. Both borders of sterilized segments were cut off, and the rest was incubated at 28±1 ˚C on SDA medium supplemented with 200µg/mL ampicillin and 200µg/mL streptomycin (to inhibit the bacterial growth) until the mycelium or colony originating from the newly formed surface of the segments appeared. The mycelium was purified in the same condition. Another segment of the same origin without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the segment surface. Efficiency of surface sterilization was checked by inoculation the surface imprints of segments and pieces. The purified endophytic fungi were numbered and transferred to fresh SDA slants separately and were kept at 4 ˚C after being cultured at 28±1 ˚C for 7 days.[50-52]

- **Cultivation of endophytic fungi**

The fresh mycelia (grown on SDA plates at 28±1 ˚C for 3–6 days) of endophytic fungi with different morphology were inoculated into 250-mL flasks containing 100 mL of improved Czapek's

broth medium (sucrose, 30 g; $NaNO_3$, 3 g; K_2HPO_4, 1 g; yeast extract, 1 g; KCl, 0.5 g; $MgSO_4$.7H2O, 0.5 g; $FeSO_4$, 0.01 g; H_2O, 1000 mL; autoclaved at 121°C for 20 min), followed by incubation in a shaking incubator at 140 rpm for 15 days at 28°C. The culture broth of each endophyte was centrifuged at 10000 rpm for 10 min and filtered using a filter with a 0.44µm nylon membrane under vacuum at ~23 °C to remove mycelium. The filtrate volume was reduced using rotary flash evaporator. Concentrated filtrate was then stored at 4 °C until use within 24 hours.[49]

7.2 PRIMARY SCREENING

- **Giant colony technique[53]**

- **Principle**

The fungal cultures are inoculated onto the central areas of petri plates containing a nutritious agar medium, or they are streaked in a narrow band across the centers of the plates. The plates are then incubated until growth and, possibly, sporulations have occurred. Strains of microorganisms to be tested for possible sensitivity to the antibiotics (the test organism) are then streaked from the edges of the plates up to but not touching the fungal growth. The plates are further incubated to allow growth of the test organism, and the distance over which the growth of test organism has been inhibited by antibiotic in the vicinity of the fungi is then measured in millimeters. Obviously, whose growth has been inhibited for a considerable distance from the fungi colony, are more sensitive to the antibiotic than those test microorganisms that can grow close to the fungi colony. Only those fungi that have produced antibiotics with interesting microbial inhibition spectra are retained for further testing.

- **Procedure**

Sauboard dextrose agar medium was prepared, sterilized and cooled. The media was poured into Petri dishes and allowed to solidify. Isolated fungi (F1 to F8) were streaked on media at straight line and incubated at 28±1°C for 4 days. The test fungi were then streaked at an angle of 90° and

incubated at 28±1°C for 24 hrs. The length of inhibition was observed if any. The fungi showing antifungal activity were further confirmed by agar diffusion method.

- **Baljet reaction** [54-55]

- **Principle**

Cardenolides give a yellow orange color with alkaline picrate solution. The yellow color reaches its intensity maximum 10 minutes after the addition of the NaOH solution and remains unchanged for about 15 min.

- **Procedure**

A portion of the filtrate was dissolved in methanol (1:1). To 2.5 mL of this solution 2.5 mL aqueous picric acid (1.3 % w/v) was added. Entire content was diluted with 0.6 mL distilled water and made alkaline with 0.6 mL NaOH. The absorbance of the resulting solution was measured at 490 nm for 15 minute at 1 minute time interval. Fungi showing best color reaction in baljet test were cultivated in large scale by similar incubation conditions. The supernatant was collected after centrifugation and lyophilized. The lyophilized product was further studied for biological activities.

7.3 BIOLOGICAL SCREENING

- ***In vitro* antifungal study**[56]

The screening of antifungal activity for the determination of the minimal inhibitory concentration (MIC) was achieved by an adaptation of the agar streak dilution method based on radial diffusion. Suspensions of the microorganism were prepared to contain approximately 108 cfu/mL, and the plates containing agar medium were inoculated (100 µL; spread on the surface). The sample (50 µL F3 and F4 metabolites) of varying concentrations were placed in a hole (3 mm depth, 6 mm diameter) made in the nutritious agar and allowed to diffuse in the media for 2 h (at 4°C). Under the same

conditions, different solutions of cycloheximide were used as standards. The MIC was considered to be the lowest concentration of the tested sample able to inhibit the growth of fungi after 48 h. The diameters of the inhibition zones corresponding to the MICs were measured using a ruler, with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times (three different plates) and the average was considered. A control using only inoculation was also carried out.

- **Animals**[57]

All the experiments were carried out using male Swiss Albino mice (25-30g) and Wistar Rats (150-200 gm) procured from the animal house, IRT Perundurai Medical College, Erode, India. On arrival the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temp of 24±2 ˚C and 30-70% relative humidity. A 12:12 h light: dark cycle was followed. All animals were allowed to free access to water and fed with standard commercial rat chaw pallets. All the experimental procedures and protocols used in this study were reviewed by the institutional animal ethics committee (Regd. no: 688/02/C-CPCSEA date: 21-02-2002) and were in accordance with the guidelines of the CPCSEA.

- **Acute oral toxicity studies**[57]

An acute oral toxicity study was performed according to OECD-423 guidelines (acute toxic class method). Swiss albino mice (25-30g) (n=3) of either sex selected by random sampling technique are employed in this study. The animals were fasted for 4 h with free access to water only. F3 and F4 were administered orally at a dose of 5mg/kg (suspended in 0.5% CMC) initially and mortality is observed for 3 days. If mortality is observed in 2/3 or 3/3 animals, then the dose administered is considered as toxic dose. However, if the mortality is observed in only one animal out of 3 animals then the same dose is repeated again to confirm the toxic effect. If mortality is not observed, the

procedure is repeated with higher doses such as 50, 300 and 2000mg/kg.

- ***In vivo* cardiotonic activity[4, 58]**

Male Wistar rats were divided in three groups (n=2). Group 1 served as control and orally received 0.5% CMC solution only. Group 2 animals were orally fed with 1000mg/kg of F3 metabolites suspended in 0.5% CMC solution and Group 3 animals were orally fed with 1000mg/kg of F4 metabolites suspended in 0.5% CMC.

The animals were anaesthetized with intra muscular injection of diazepam (1mg/kg) one hour after giving dose. ECG was recorded using electrocardiogram. The red and black electrodes were applied on right and left hind arm near femoral vein respectively. The white electrode was applied on the left forearm near thoracic cavity. All leads were established by surface electrodes to minimize injury to the animal. The output signals of these systems were recorded at 50-150 Hz.

- ***In Vivo* Antidiabetic Study[57, 59-60]**

- **Induction of diabetes**

Male Wistar rats weighing 150–200 g were used for this study. For the induction of diabetes, rats were kept on fasting for 24 hours prior to alloxan injection. On the day of administration, alloxan tetrahydrate was freshly dissolved in 0.01 M (pH 4.5) citrate buffer and subcutaneous injection was given at the dosage of 250 mg/kg. Blood glucose concentration was checked by the Glucometer after 1 week of alloxan injection. The animals with glucose concentration exceeding 250 mg/dl were considered diabetic.[59]

- **Experimental Design**

All the rats were divided into 5 groups:

Group 1	Control animals received citrate buffer injection only
Group 2	Diabetic animals received alloxan injection
Group 3	Diabetic animals orally fed with 8 mg/kg of gliclazide suspended in 0.5% carboxymethyl cellulose (CMC) solution in two divided doses orally for 15 days
Group 4	Diabetic animals orally fed with 500mg/kg of F3 metabolites suspended in 0.5% (CMC) solution in two divided doses for 15 days
Group 5	Diabetic animals orally fed with 1000mg/kg of F3 metabolites suspended in 0.5% (CMC) solution in two divided doses for 15 days

All groups except group 1 received their respective solutions through cannula. Group 2 received vehicle (0.5% CMC solution) only.[60]

- **Body weight**

The body weights of the animals were measured before and after the study period.[60]

- **Biochemical analysis**

a) Collection of serum

The animals were fasted overnight and the blood samples were withdrawn from retro-orbital plexus under light ether anesthesia without any anticoagulant and allowed to clot for 10 minutes at room temperature. It was centrifuged at 2500 rpm for 20 minutes. The serum obtained was kept at 4ºC until used.[57]

b) **Estimation of Fasting Glucose**

In vitro quantitative determination of the activity of glucose in serum was done using enzymatic kit (Beacon Diagnostics Pvt. Limited.).[57]

→ **Principle:**

Enzymatic colorimetric determination of glucose according to the following reactions:

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red quinone} + 4H_2O$$

→ **Procedure**

Pipette in the tubes labeled as follows;

	Blank	Standard	Sample
Working Reagent	2 ml	2 ml	2 ml
Distilled Water	10 µl	-	-
Standard	-	10 µl	-
Serum Sample	-	-	10 µl

Mix and read the optical density (OD) at 356 nm after 10 minutes incubation. The final color is stable for at least 1 hour.

→ **Calculations**

Fasting Glucose (mg/dl) = Abs. of sample/ Abs. of Std. X 100

c) **Estimation of total protein (Biuret method)**

In vitro quantitative measurement of total protein concentration in serum was done by using kit (Span diagnostics (India) Pvt. Ltd.).

→ **Principle**

Peptide bonds in protein react with cupric ion in alkaline solutions to form a colored chelate, the absorbance of which is measured at 578 nm. The Biuret reagent contains sodium-potassium tartarate to complex cupric ions and maintains their solubility at alkaline pH. Absorbance data is proportional to

protein concentration.

Biuret reagent: Copper sulphate, Sodium hydroxide, Sodium-potassium tatarate, Surfactant.

Protein Standard: 6.5 mg/dl

→ **Procedure**

Pipette in the tubes labeled as follows;

	Blank	Standard	Test
Serum	-	-	30 µl
Total Protein standard	-	30 µl	-
Biuret Reagent	3.0 ml	3.0 ml	3.0 ml

Mix well and incubate at 37ºC or at room temperature for 5 minutes. Read the absorbance at 578 nm against reagent blank.

→ **Calculations:**

Total Protein = Abs. of Test/ Abs. of Std. X 6.5[57]

d) Estimation of total lipids (Sulphophosphovanillin method)

→ **Principle**

Lipids react with vanillin in the presence of sulfuric and phosphoric acid to form a pink colored complex.

→ **Reagents**

1. Total lipid standard: 1000 mg/dL

   It is prepared by dissolving 1.0 gm of olive oil in chloroform.

2. Color reagent (Phosphovanillin) is prepared by mixing

   a. 0.61 gm/dl vanillin : 350 ml

   b. Orthophosphoric acid : 600 ml

   c. Distilled water : 50 ml

3. Concentrated sulfuric acid (AR)

→ **Procedure**

Pipette in the tubes labeled as follows;

	Test	Standard
Solution 1	-	0.05 ml
Serum	0.05 ml	-
Reagent 3	2.0 ml	2.0 ml

Mixed thoroughly and plugged with cotton wool. Then it was kept in a boiling water bath for 10 minutes. Then cooled in cold water bath and again pipetted into dry test tubes as follows:

	Test	Std.	Blank
From above solution	0.10 ml	0.10 ml	-
Reagent 3	-	-	0.10 ml
Coloring reagent	2.5 ml	2.5 ml	2.5 ml

Mixed thoroughly and kept at room temperature (25ºC ± 5ºC) for 15 minutes. Read absorbance of test and standard against blank in a dry cuvette at 546 nm.[57]

→ **Calculations**

Serum Total Lipid (mg/dl) = O.D. of Test/ O.D. of Std. X 100

- **Statistical analysis**

All the values of body weight and biochemical estimations were expressed as mean ± standard error of mean (S.E.M.) and analyzed by one-way ANOVA followed by Turkey-Kramer Multiple Comparison Test. Differences between groups were considered significant at $P < 0.01$ levels.

***In Vitro* Cytotoxic Study (Sulphorodamine B, SRB assay)[61]**

- **Principle**

SRB is a bright pink aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds to basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude.

- **Media**

→ DMEM (Dulbecoo's Modified Eagels medium, high glucose)

→ FBS (Fetal Bovine Serum)

- **Glass wares and plastic wares**

96-well microtiter plate, Tissue culture flasks, Falcon tubes, Reagent bottles

- **Cancer cell lines**

hep 2, mcf 7, hela and hek (National Centre For Cell Science, Pune)

- **Procedure**

1. The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 10^5 cells/ml using medium containing 10% new born sheep serum.
2. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added.
3. After 24 hours, when a partial monolayer was formed, 100µl of different compound (F3 and F4 metabolites) concentrations were added to the cells in microtitre plates. The plates were then incubated at 37^{o}C for 72 hours in 5% CO_2 incubator and microscopic examination was carried

out and observations recorded every 24 hours.

4. After 72 hours, 25µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form a over all concentrations 10%.

5. The plates were incubated at 4^{o}C for one hour.

6. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried.

7. The air-dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried.

8. 100µl of 10mM Tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 minutes.

9. The absorbance was measured using ELISA plate reader at a wavelength of 620 and 540nm.

10. The percentage growth inhibition was calculated using following formula,

%cell inhibition= 100-{(At-Ab)/ (Ac-Ab)} x100

Where, At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

8. RESULTS AND DISCUSSION

8.1 ISOLATION OF ENDOPHYTIC FUNGI

Total 8 endophytic fungi were isolated from *Nerium oleander* and given code as follows:

Stem: F5

Leaf: F2 and F6

Root: F1, F3, F4, F7 and F8

Figure 8.1: F1

Figure 8.2: F2

Figure 8.3: F3

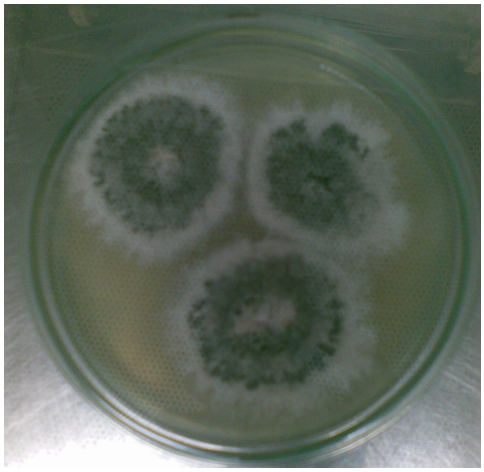


Figure 8.4: F4

Figure 8.5: F5

Figure 8.6: F6

Figure 8.7: F7

Figure 8.8: F8

8.2 PRIMARY SCREENING

- **Giant colony technique**

The endophytic fungi F3 and F4 showed antifungal activity against some test fungi. The results are shown in the table 8.1.

Table 8.1 Inhibition of growth of test fungi by endophytic fungi

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
Arthrobotrys oligospora (NCIM 1246)	N	N	Y	Y	N	N	N	N
Aspergillus niger (NCIM 1207)	N	N	Y	Y	N	N	N	N
Auricularia polytricha (NCIM 1303)	N	N	N	N	N	N	N	N
Candida albicans (NCIM 3484)	N	N	Y	Y	N	N	N	N
Chaetomella raphigera (NCIM 1231)	N	N	Y	Y	N	N	N	N
Monilinia fruticola (NCIM 1011)	N	N	N	N	N	N	N	N
Sacchromyces cerevisae (ATCC 204508)	N	N	Y	Y	N	N	N	N

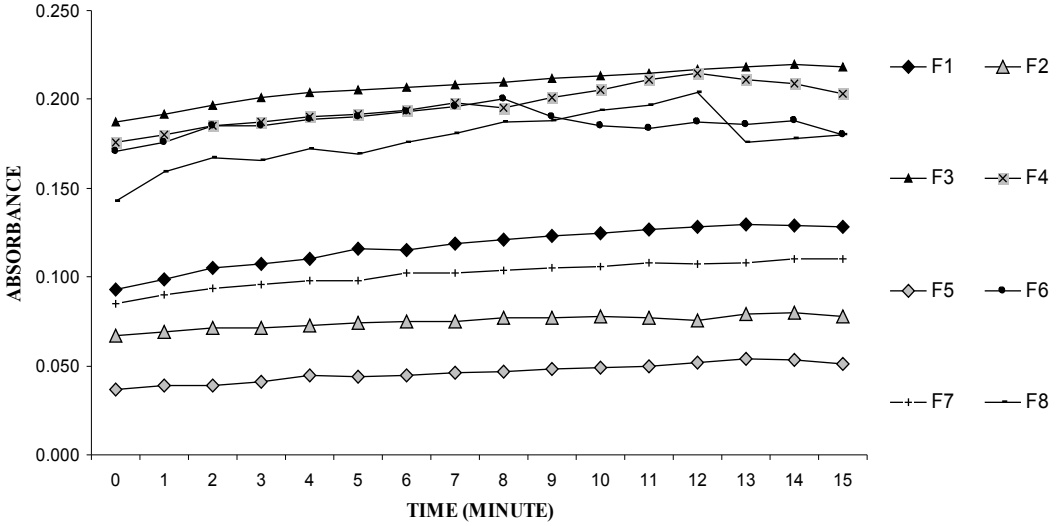
N→no inhibition; Y→inhibition of growth of test fungi

Endophytic fungi F3 and F4 were thus cultivated on large scale for *in vitro* antifungal activity.

- **Baljet reaction**

The absorbances of the reaction product of Baljet test at 490 nm for 15 minute at 1 minute time interval are as follows:

Figure 8.9: Baljet reaction



The figure shows that F3 and F4 metabolites showed best color reaction in baljet test. They were thus cultivated on large scale for biological screening.

8.3 BIOLOGICAL SCREENING

Endophytic fungi F3 and F4 were cultivated in large scale for biological screening. The medium and incubation conditions were described in the procedure. After incubation period the mycelium was separated by centrifugation. Resulting supernatant was freeze dried and the freeze dried product was used for the study.

Figure: 8.10 Freeze Dried Powder of Endophytic Fungi supernatant

F3 **F4**

- ***In vitro* antifungal study**

The endophytic fungi F3 and F4 were screened for their antifungal properties against *A. oligospora*, *A. niger*, *A. polytricha*, *C. albicans*, *C. raphigera*, *M. fruticola* and *S. cerevisae*. The minimal inhibitory concentration (MIC) values for the tested fungi were determined as an evaluation of the antimicrobial activity of the tested freeze dried supernatant of the endophytic fungal culture. The results are shown in table 8.2. The response for each microorganism tested was different. The endophytic fungi revealed antifungal activity showing different selectivity and MICs for each test fungi. The pure active compound cycloheximide presented lower MICs than the endophytic fungi.

Figure 8.11: Antifungal activity of F3 metabolites against *Arthrobotrys oligospora*

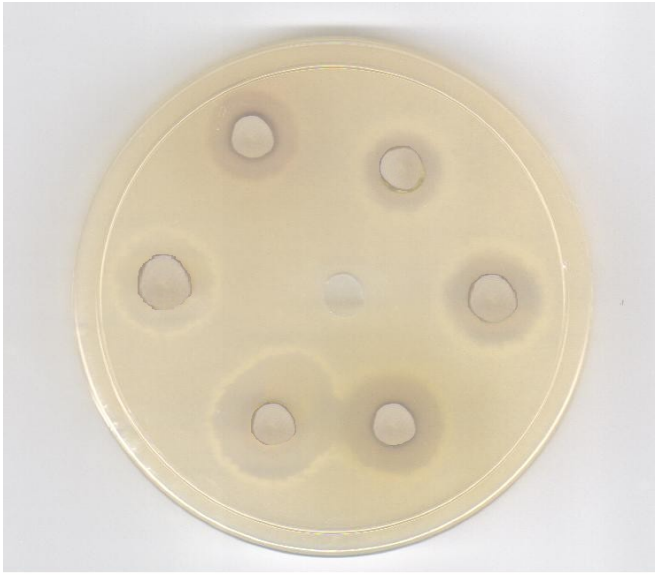


Table 8.2: Antifungal activity of metabolites of F3 and F4, and Cycloheximide

Test Organism	MIC (mg/ml)*		
	F3	F4	Cycloheximide
Arthrobotrys oligospora	13	13.5	0.50
Aspergillus niger	13.5	12.5	1.00
Candida albicans	15	14.5	0.50
Chaetomella raphigera	14	13	0.50
Sacchromyces cerevisae	12.5	12	0.75

*- calculated as freeze dried powder of the fungal culture

- **Acute oral toxicity studies**

F3 and F4 metabolites did not cause any mortality up to 2000 mg/kg and were considered as safe. No lethality or any toxic reactions were found up to the end of the study period.

- ***In vivo* cardiotonic activity:**

There was no significant change in the ECG pattern of F3 metabolite administered group and a slight prolongation of PR interval (delayed atrioventricular conduction) in F4 metabolite administered group. It confirms a mild cardiotonic activity of F4 metabolite on rat heart.

Figure 8.12: ECG of Group 1 (control group)

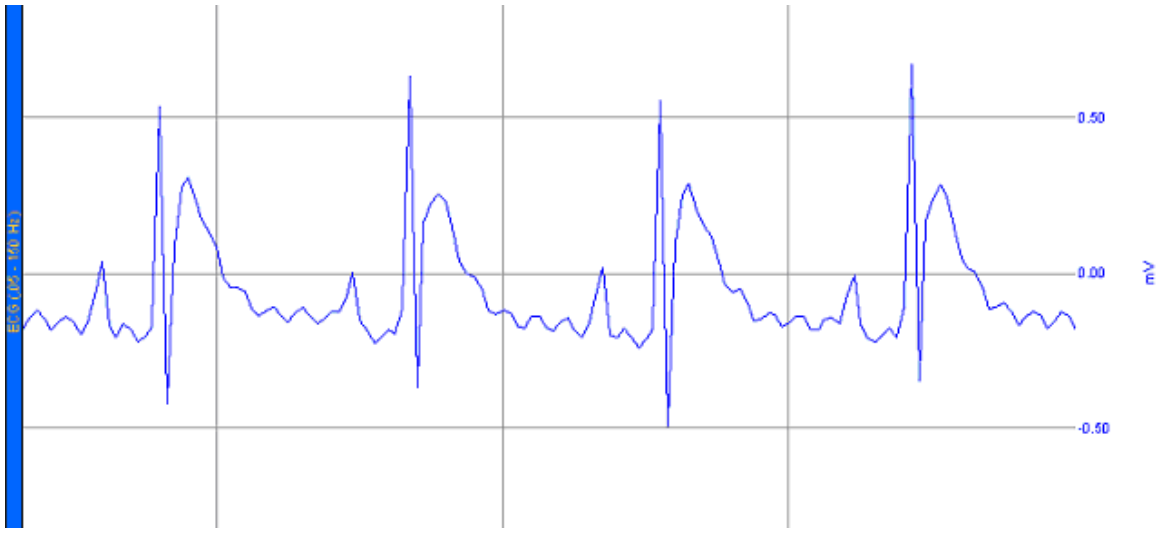


Figure 8.13: ECG of Group 2 (F3 metabolite administered group)

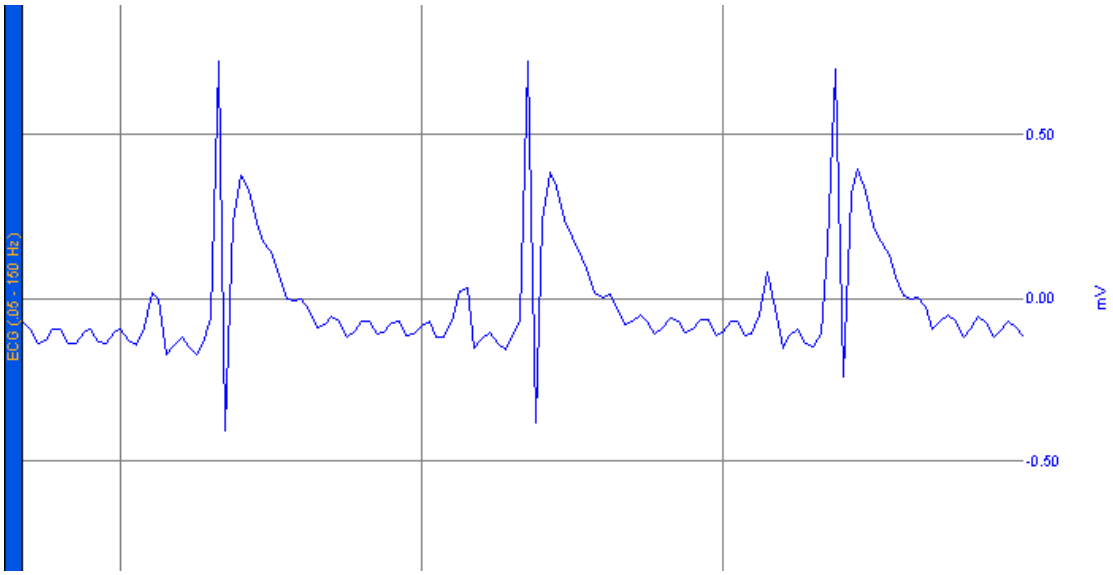
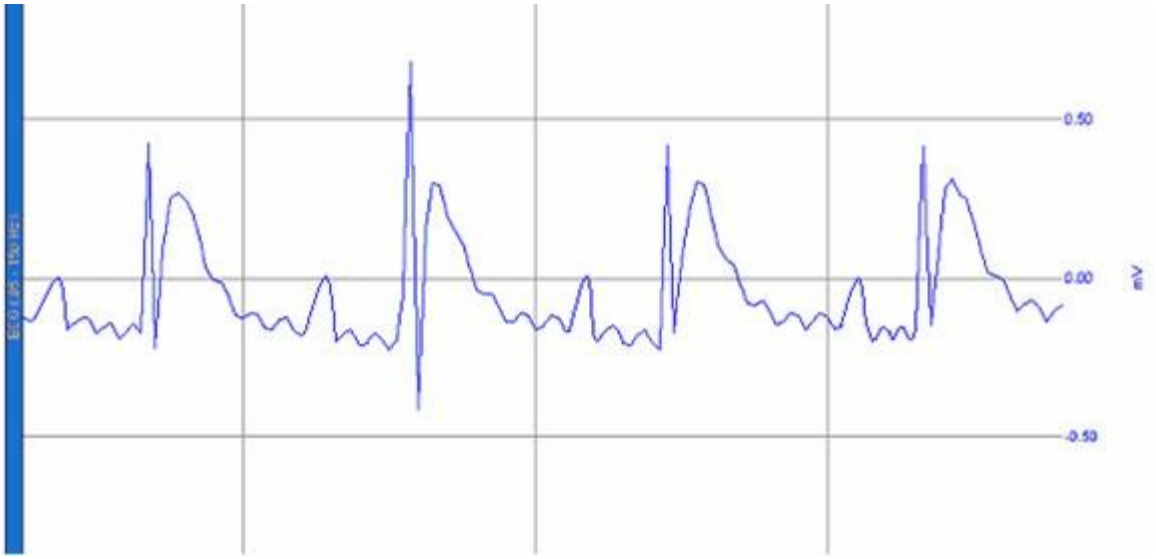


Figure 8.14 ECG of Group 3 (F4 metabolite administered group)



- ***In Vivo*** **Antidiabetic Study**

Figure 8.15: Oral Drug Administration to Rat

- **Body weight change**

Treatment with F3 reversed the weight loss in diabetic rats. It is given in table.

Table 8.3: Average body weight (g) of experimental groups (n=6)

	Initial	Final
Group 1	187.00 ± 4.06	190.33 ± 4.27
Group 2	182.17 ± 2.60	171.33 ± 2.66
Group 3	183.67 ± 2.49	191.33 ± 2.80
Group 4	184.67 ± 3.06	197.83 ± 3.43
Group 5	183.17 ± 2.89	190.67 ± 3.21

- **Biochemical analysis**

The results of the present investigation are presented in Table 8.4-8.7. In all groups before alloxan administration, the basal levels of blood glucose of the rats were not significantly different. However, 7 days after alloxan administration, blood glucose levels were significantly higher in the experimental rats selected for the study. Table presents the changes in body weight, fasting blood

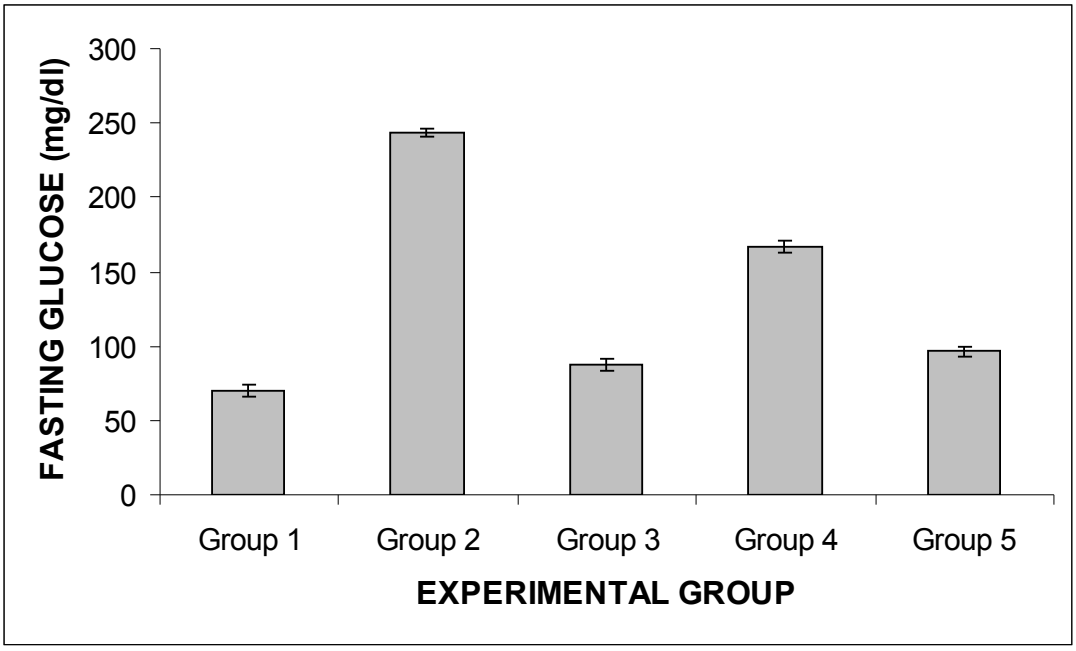
glucose, total lipids and total proteins in normal and experimental diabetic rats. The body weights in the F3 metabolite-treated and gliclazide-treated groups increased significantly ($P<0.001$) at the end of week 2 when compared with the diabetic control group. A significant antihyperglycemic effect was observed in the F3-treated and gliclazide-treated groups ($P<0.001$). The serum total protein values were significantly decreased in gliclazide-treated group ($P<0.01$) while there is no significant decrease in F3 treated group as compared with the diabetic control group. The values of serum total lipids shows a significant decrease in the F3-treated and gliclazide-treated groups ($P<0.001$).

Table 8.4: Serum fasting glucose of experimental groups*

	Absorbance of test	**Absorbance of standard**	**Fasting glucose (mg/dl)**
Group 1	0.278	0.397	70.025
Group 2	0.966	0.397	243.325
Group 3	0.347	0.397	87.406
Group 4	0.661	0.397	166.499
Group 5	0.382	0.397	96.222

*→Values are represented as mean (n=6)

Figure 8.16 Serum fasting glucose of experimental groups**



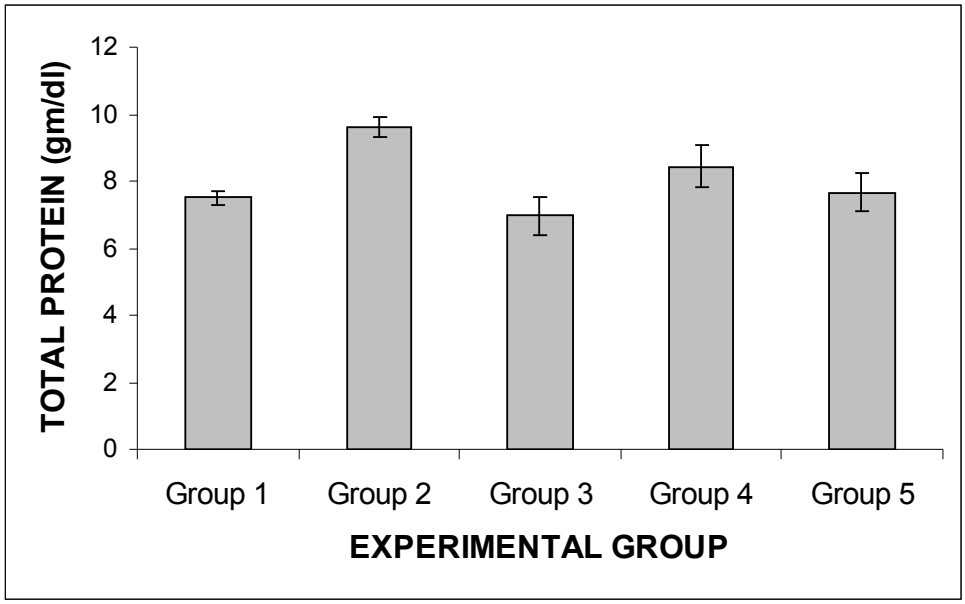
**→Values are represented as mean ± standard error of mean (n=6)

Table 8.5: Serum total protein of experimental groups*

	Absorbance of test	Absorbance of standard	Total protein (gm/dl)
Group 1	0.532	0.461	7.501
Group 2	0.681	0.461	9.602
Group 3	0.494	0.461	6.965
Group 4	0.598	0.461	8.432
Group 5	0.543	0.461	7.656

*→Values are represented as mean (n=6)

Figure 8.17 Serum total protein of experimental groups**



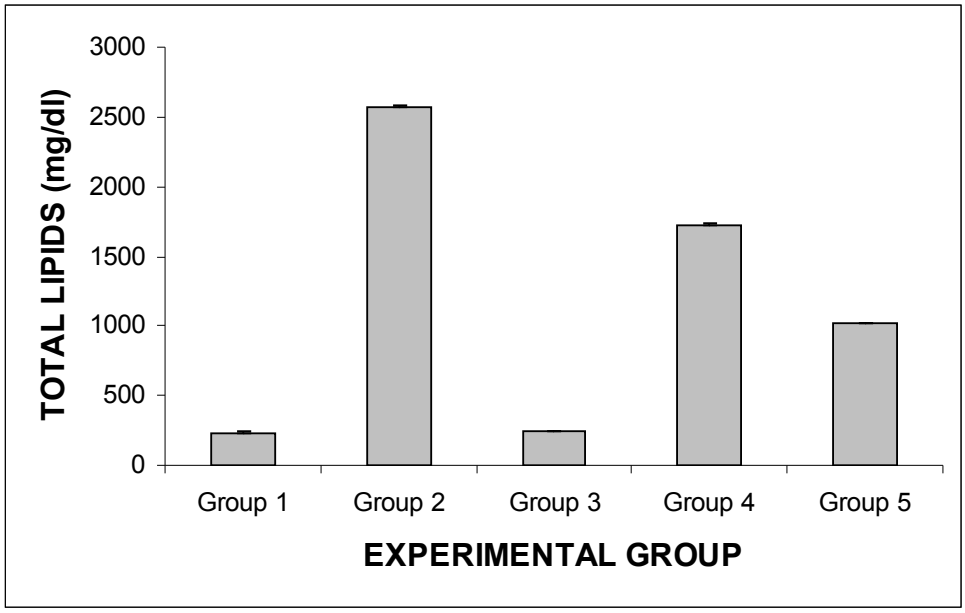
**→Values are represented as mean ± standard error of mean

Table 8.6: Serum total lipids of experimental groups*

	Absorbance of test	Absorbance of standard	Total lipids (mg/dl)
Group 1	0.074	0.032	231.563
Group 2	0.823	0.032	2571.875
Group 3	0.079	0.032	245.313
Group 4	0.553	0.032	1728.125
Group 5	0.326	0.032	1018.750

*→Values are represented as mean (n=6)

Figure 8.18: Serum total lipids of experimental groups**



**→Values are represented as mean ± standard error of mean

In Vitro Cytotoxic Study (Sulphorodamine B, SRB assay)

F3 and F4 metabolites were screened for cytotoxic activity using SRB assay. F3 metabolites showed cytotoxic effect on mcf 7 while it does not possess significant cytotoxicity against hep 2, hela and hek cell lines. F4 metabolites showed significant cytotoxicity against mcf 7 and hela cell lines while it has less significant action on hep 2 and hek cell lines. The results of SRB assay are shown in the tables (8.8-8.15) and figures (8.19-8.26):

Table 8.8: Cytotoxic Effect of F3 metabolites on HEP 2

	Absorbance		% cell inhibition	
Concentration of F3 (mg/ml)	**690 nm**	**540 nm**	**690 nm**	**540 nm**
blank	0.118	0.199	-	-
control	0.047	0.067	-	-
0.0196	0.102	0.156	77.46	67.42
0.0391	0.075	0.113	39.44	34.85
0.078	0.062	0.089	21.13	16.67
0.156	0.061	0.079	19.72	9.09
0.312	0.053	0.07	8.45	2.27
0.625	0.074	0.093	38.03	19.70
1.25	0.07	0.086	32.39	14.39
2.5	0.07	0.085	32.39	13.64
5	0.057	0.067	14.08	0.00
10	0.082	0.107	49.30	30.30

Figure 8.19: Cytotoxic Effect of F3 metabolites on HEP 2

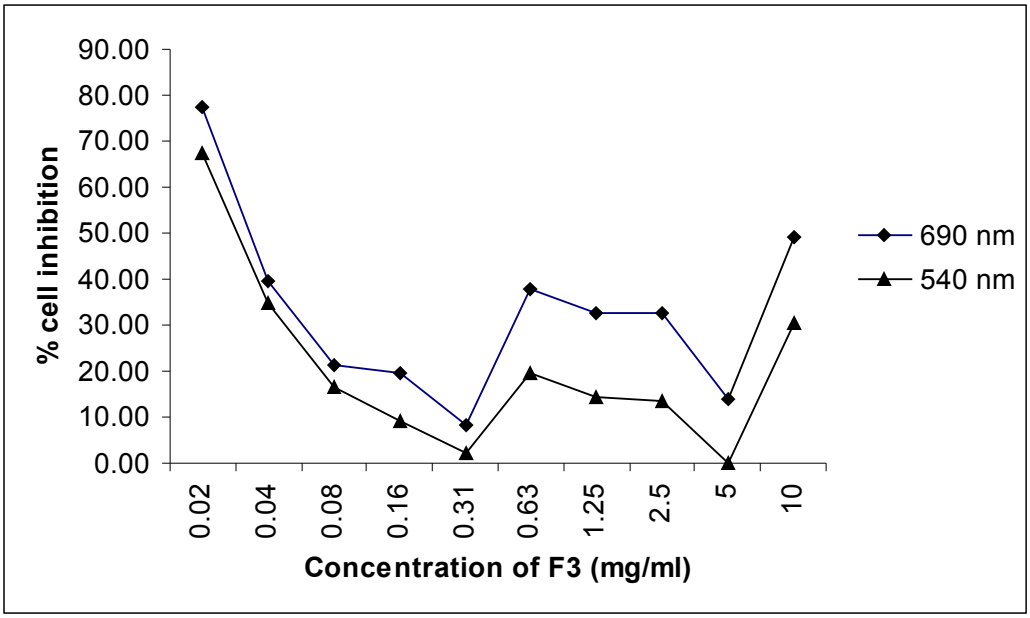


Table 8.9: Cytotoxic Effect of F4 metabolites on HEP 2				
	Absorbance		% cell inhibition	
Concentration of F4 (mg/ml)	690 nm	540 nm	690 nm	540 nm
blank	0.124	0.226	-	-
control	0.056	0.083	-	-
0.0196	0.103	0.168	69.12	59.44
0.0391	0.084	0.151	41.18	47.55
0.078	0.22	0.218	241.18	94.41
0.156	0.093	0.152	54.41	48.25
0.312	0.065	0.097	13.24	9.79
0.625	0.094	0.15	55.88	46.85
1.25	0.063	0.084	10.29	0.70
2.5	0.081	0.114	36.76	21.68
5	0.074	0.087	26.47	2.80
10	0.066	0.079	14.71	-2.80

Figure 8.20: Cytotoxic Effect of F4 metabolites on HEP 2

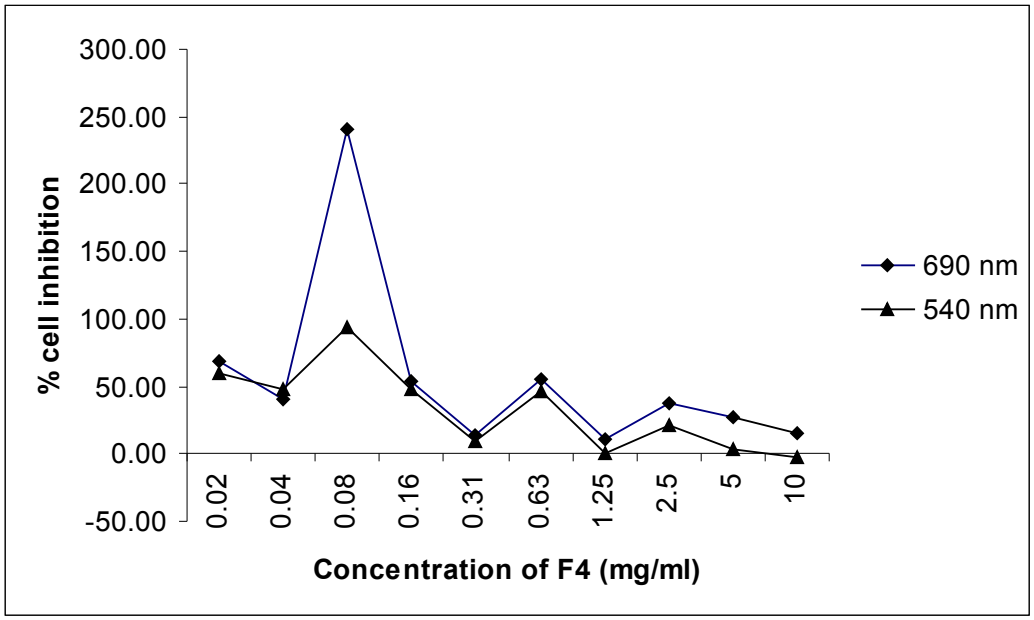


Table 8.10: Cytotoxic Effect of F3 metabolites on MCF 7				
	Absorbance		% cell inhibition	
Concentration of F3 (mg/ml)	690 nm	540 nm	690 nm	540 nm
blank	0.045	0.051	-	-
control	0.077	0.123	-	-
0.0196	0.158	0.206	-253.13	-115.28
0.0391	0.142	0.188	-203.13	-90.28
0.078	0.129	0.174	-162.50	-70.83
0.156	0.116	0.154	-121.88	-43.06
0.312	0.108	0.139	-96.88	-22.22
0.625	0.091	0.126	-43.75	-4.17
1.25	0.077	0.111	0.00	16.67
2.5	0.062	0.097	46.88	36.11
5	0.047	0.082	93.75	56.94
10	0.026	0.068	159.38	76.39

Figure 8.21: Cytotoxic Effect of F3 metabolites on MCF7

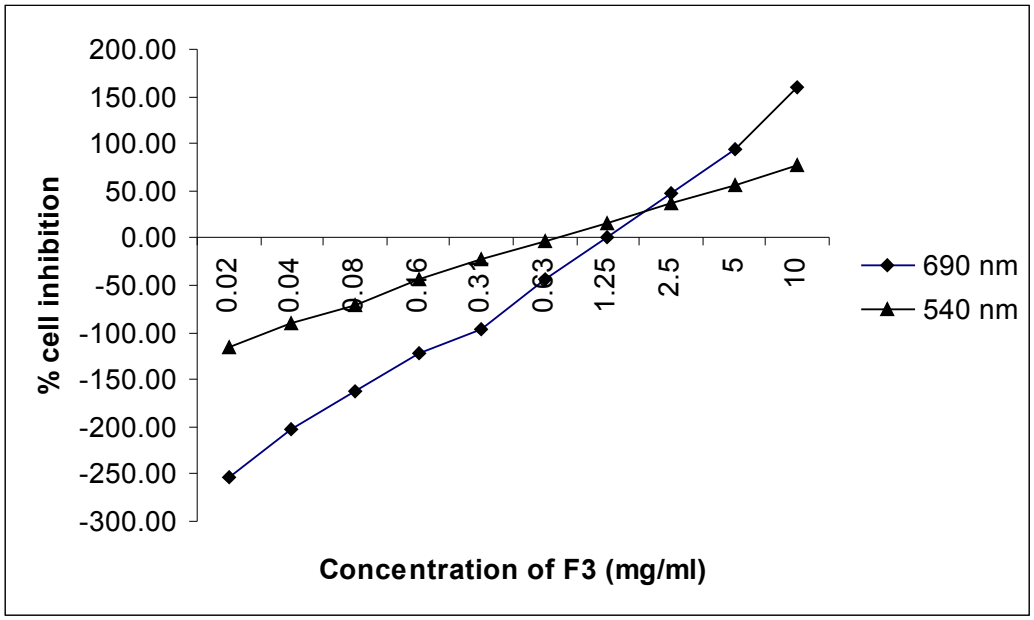


Table 8.11: Cytotoxic Effect of F4 metabolites on MCF 7				
	Absorbance		% cell inhibition	
Concentration of F4 (mg/ml)	690 nm	540 nm	690 nm	540 nm
blank	0.049	0.07	-	-
control	0.186	0.245	-	-
0.0196	0.124	0.231	45.26	8.00
0.0391	0.098	0.213	64.23	18.29
0.078	0.086	0.196	72.99	28.00
0.156	0.069	0.175	85.40	40.00
0.312	0.055	0.152	95.62	53.14
0.625	0.044	0.125	103.65	68.57
1.25	0.031	0.108	113.14	78.29
2.5	0.025	0.075	117.52	97.14
5	0.011	0.055	127.74	108.57
10	0.001	0.035	135.04	120.00

Figure 8.22: Cytotoxic Effect of F4 metabolites on MCF 7

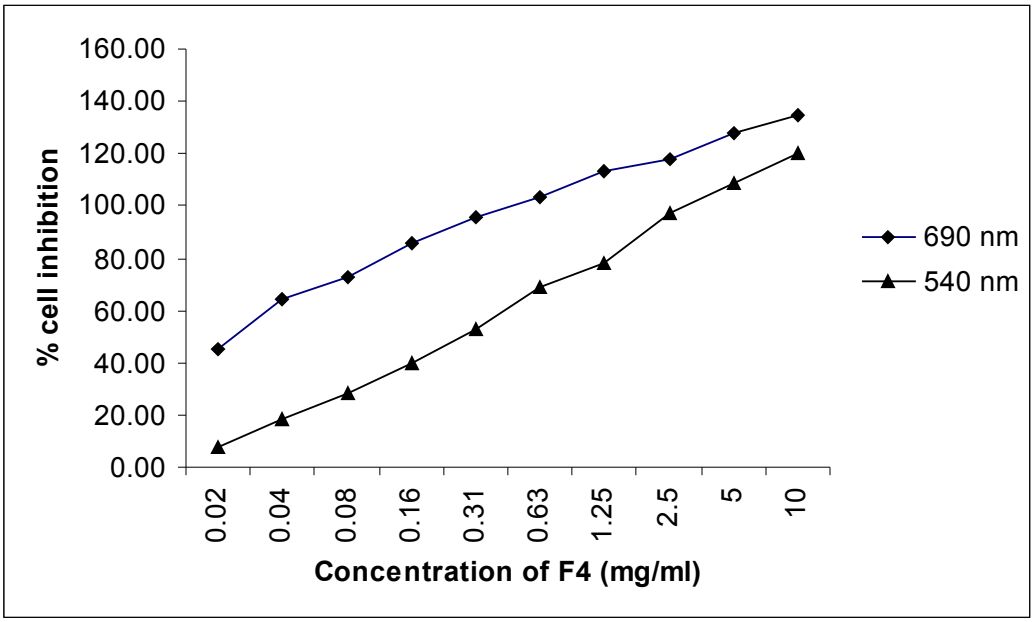


Table 8.12: Cytotoxic Effect of F3 metabolites on HELA				
	Absorbance		% cell inhibition	
Concentration of F3 (mg/ml)	690 nm	540 nm	690 nm	540 nm
blank	0.072	0.112	-	-
control	0.12	0.201	-	-
0.0196	0.19	0.308	-145.83	-120.22
0.0391	0.122	0.206	-4.17	-5.62
0.078	0.232	0.298	-233.33	-108.99
0.156	0.159	0.234	-81.25	-37.08
0.312	0.109	0.187	22.92	15.73
0.625	0.086	0.111	70.83	101.12
1.25	0.059	0.078	127.08	138.20
2.5	0.103	0.151	35.42	56.18
5	0.057	0.07	131.25	147.19
10	0.071	0.086	102.08	129.21

Figure 8.23: Cytotoxic Effect of F3 metabolites on HELA

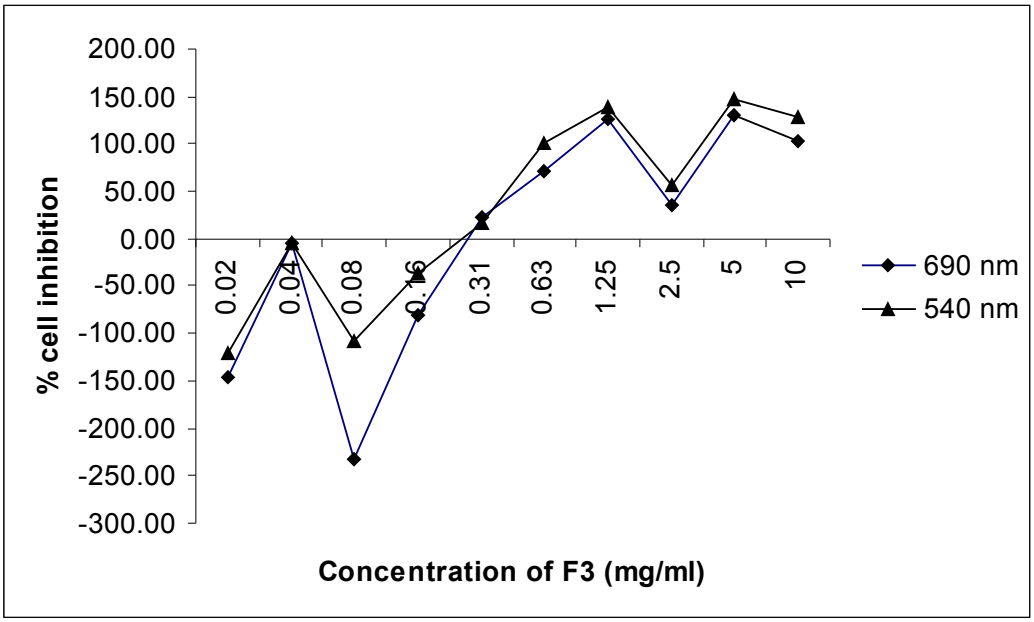


Table 8.13: Cytotoxic Effect of F4 metabolites on HELA				
	Absorbance		% cell inhibition	
Concentration of F4 (mg/ml)	690 nm	540 nm	690 nm	540 nm
blank	0.058	0.061	-	-
control	0.227	0.263	-	-
0.0196	0.154	0.242	43.20	10.40
0.0391	0.141	0.221	50.89	20.79
0.078	0.129	0.171	57.99	45.54
0.156	0.112	0.164	68.05	49.01
0.312	0.098	0.144	76.33	58.91
0.625	0.089	0.141	81.66	60.40
1.25	0.074	0.099	90.53	81.19
2.5	0.061	0.099	98.22	81.19
5	0.048	0.071	105.92	95.05
10	0.035	0.066	113.61	97.52

Figure 8.24: Cytotoxic Effect of F4 metabolites on HELA

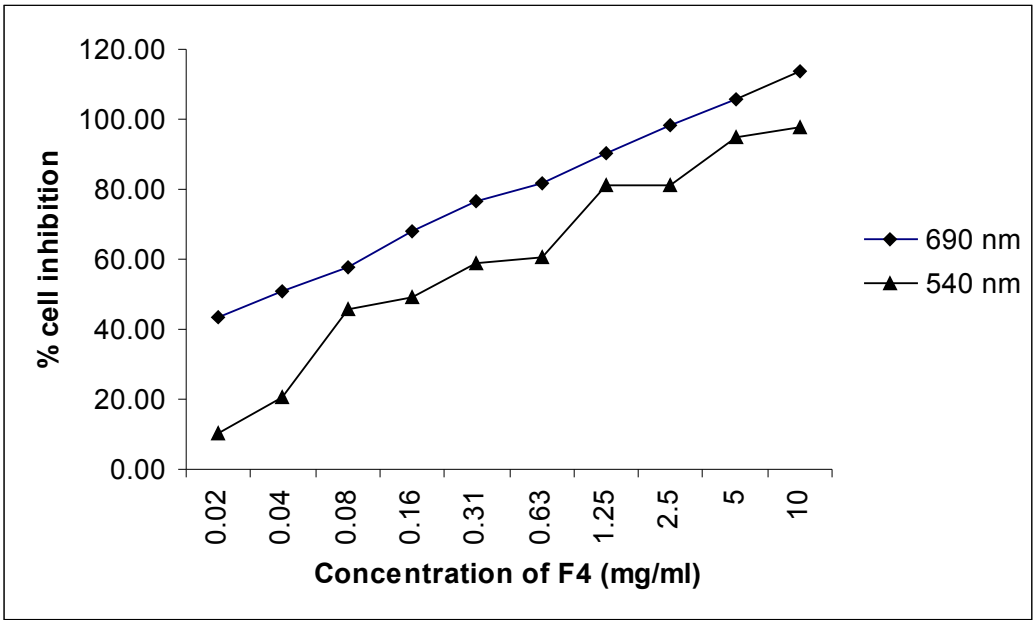


Table 8.14: Cytotoxic Effect of F3 metabolites on HEK				
	Absorbance		% cell inhibition	
Concentration of F3 (mg/ml)	690 nm	540 nm	690 nm	540 nm
blank	0.076	0.12	-	-
control	0.287	0.804	-	-
0.0196	0.174	0.261	53.55	79.39
0.0391	0.11	0.192	83.89	89.47
0.078	0.239	0.374	22.75	62.87
0.156	0.069	0.112	103.32	101.17
0.312	0.186	0.238	47.87	82.75
0.625	0.011	0.124	130.81	99.42
1.25	0.065	0.1	105.21	102.92
2.5	0.052	0.071	111.37	107.16
5	0.056	0.067	109.48	107.75
10	0.068	0.098	103.79	103.22

Figure 8.25: Cytotoxic Effect of F3 metabolites on HEK

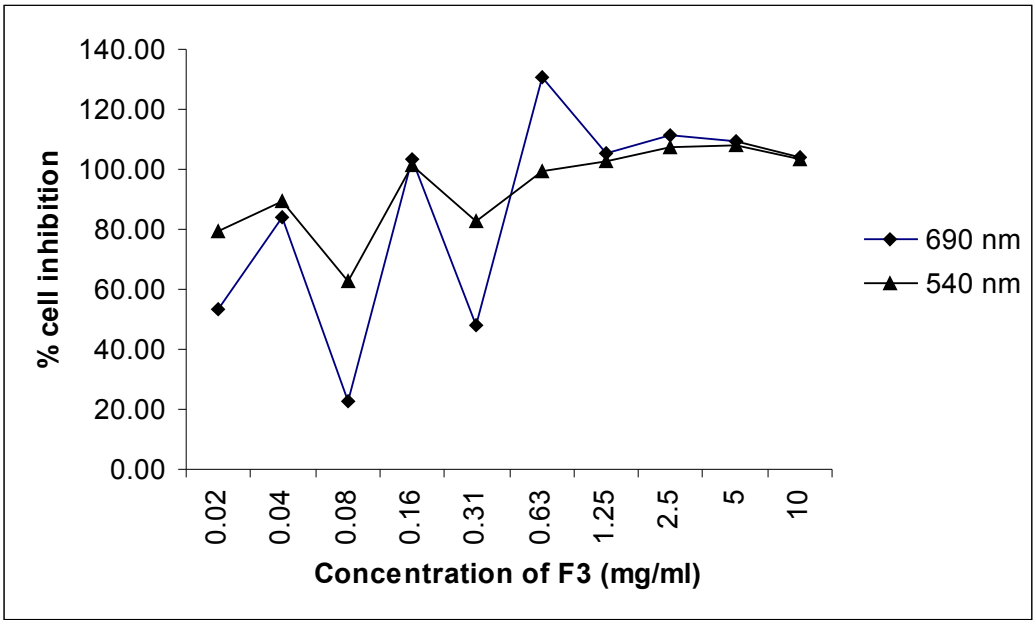
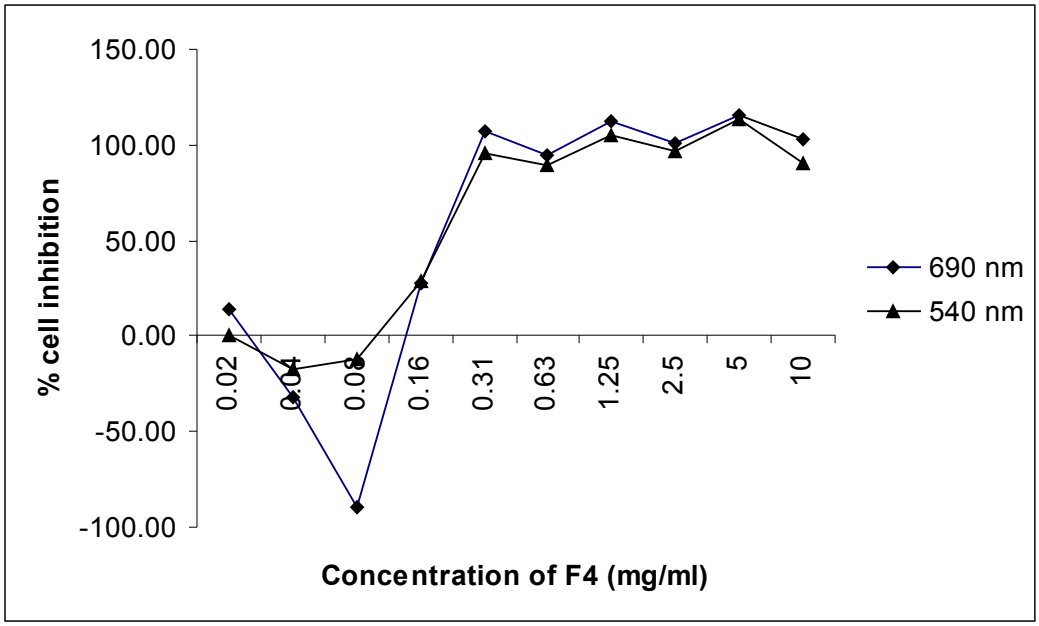


Table 8.15: Cytotoxic Effect of F4 metabolites on HEK				
	Absorbance		% cell inhibition	
Concentration of F4 (mg/ml)	690 nm	540 nm	690 nm	540 nm
Blank	0.08	0.105	-	-
Control	0.244	0.371	-	-
0.0196	0.221	0.37	14.02	0.38
0.0391	0.297	0.416	-32.32	-16.92
0.078	0.39	0.404	-89.02	-12.41
0.156	0.198	0.296	28.05	28.20
0.312	0.068	0.116	107.32	95.86
0.625	0.089	0.132	94.51	89.85
1.25	0.059	0.093	112.80	104.51
2.5	0.078	0.115	101.22	96.24
5	0.054	0.07	115.85	113.16
10	0.075	0.131	103.05	90.23

Figure 8.26: Cytotoxic Effect of F4 metabolites on HEK


150.00
100.00
50.00
0.00
-50.00
-100.00
% cell inhibition
0.02
0.04
0.08
0.16
0.31
0.63
1.25
2.5
5
10
Concentration of F4 (mg/ml)
690 nm
540 nm

9. SUMMARY

Total 8 (one from stem, two from leaf and five from root) endophytic fungi were isolated from *Nerium oleander*. They were screened for antifungal, cardiotonic, antidiabetic and cytotoxic activity.

The fungi were cultivated using improved Czapek's broth medium. Two fungi (F3 and F4) were found to produce antifungal metabolites and inhibited the growth of *Arthrobotrys oligospora* (NCIM 1246), *Aspergillus niger* (NCIM 1207), *Candida albicans* (NCIM 3484), *Chaetomella raphigera* (NCIM 1231), and *Sacchromyces cerevisae* (ATCC 204508) whereas it showed poor activity against *Auricularia polytricha* (NCIM 1303) and *Monilinia fruticola* (NCIM 1011).

An acute oral toxicity study was performed to determine the dose for cardiotonic and antidiabetic screening. Signs of toxicity were not observed up to 2000mg/kg of body weight.

F3 was not having any significant change in ECG pattern while F4 prolonged the PR interval in the ECG, indicating its mild cardiotonic activity on rat.

There was a significant recovery of fasting glucose, total lipids and body weights in diabetic models (alloxan induced)of rats treated with F3, while it does not showed significant change in serum protein level. P values were less than 0.001 in case of body weight, fasting and total lipids, while it was not significant for total proteins. The effect was dose dependant.

F3 possessed cytotoxic effect on mcf 7 while it was not having significant cytotoxicity against hep 2, hela and hek cell lines. F4 showed significant cytotoxicity against mcf 7 and hela cell lines while it showed less significant action on hep 2 and hek cell lines.

10. CONCLUSION

Endophytic fungi were isolated from *Nerium Oleander* Linn (Apocynaceae). They have produced metabolites with antifungal, cardiotonic, antidiabetic and cytotoxic activity without causing any toxic effect. Anvirzel™ is an aqueous extract of the plant *Nerium oleander* which has been utilized to treat patients with advanced malignancies. Present study has shown that endophytic fungi have a potential to replace the use of host plant therapeutically.

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